Broadly protective nanoparticle-based mucosal vaccine against influenza virus infection

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Cover illustration: "Dendritic cells targeted by nanoparticles"

by Anneli Strömberg

Nanoparticles (green), early endosomes (red), late endosomes (white), nuclei (blue)

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"If you think research is expensive, try disease"

Mary W. Lasker

(1900-1994)

American health activist

and philanthropist;

founder of the Lasker Foundation.

ABSTRACT

Influenza is one of the major viral diseases affecting humans and it is responsible for three to five million cases of severe illness and about 250.000 to 500.000 deaths each year worldwide. A vaccine against pandemic influenza infection is much warranted and the most effective measure to reduce the risk of a global spread of novel emerging influenza strains. While injectable vaccines require trained medical staff and carry a substantial risk of spreading contaminating infections, mucosal vaccines are easier to administer and considered safer, but, unfortunately, also less effective. However, mucosal vaccines can be made more effective by using better formulations and adjuvants. We have designed two intranasal vaccine candidates against pandemic flu, which were based on the strain-conserved M2e peptide incorporated into the CTA1-DD mucosal adjuvant. Previously, the CTA1-3M2e-DD fusion protein was found to stimulate protective immunity. Here, we attempted to further improve its vaccine qualities by incorporating it into polysaccharide or liposome nanoparticles, which were administered intranasally. Our findings clearly indicate that mucosal vaccines based on combinations of the potent CTA1-DD immunomodulator and nanoparticles provide a strong basis for future mucosal vaccine development. Finally, my thesis work conveys optimism about the possibility to develop a broadly protective mucosal influenza vaccine not only for adults, but also for young children.

Keywords: Mucosal vaccination; Influenza A virus; CTA1-DD; Nanoparticle; Targeted adjuvant; Nasal immunization; Neonatal vaccine; Universal vaccine.

SAMMANFATTNING PÅ SVENSKA

Influensa är en av våra allvarligaste virussjukdomar, som påverkar mängder av människor över hela världen. Man beräknar att 3-5 miljoner fall av allvarlig sjukdom är orsakad av influensavirus och cirka 250.000 till 500.000 dödsfall registreras varje år. Ett brett skyddande vaccin mot influensa är därför högt eftertraktat och skulle kunna skydda mot risken för en global spridning av nya influensastammar. Vanliga injicerbara vacciner kan, tyvärr, sprida annan smitta genom orena kanyler och får bara ges av medicinskt utbildad personal. Vacciner som ges via slemhinnor, som dryck eller spray däremot, är enkla och säkrare att ge, men tyvärr ofta ineffektiva. Sådana s.k mukosala vacciner kan dock göras mer effektiva genom bättre vaccinformuleringar och användandet av mer potenta immunförstärkare, s.k adjuvans. I denna studie har vi prövat att effektivisera ett fusionsprotein som baseras på att M2e-peptiden från influensa inkorporerats i vårt CTA1-DD-adjuvanssystem. Denna vaccinkandidat, CTA1-3M2e-DD, har visat sig ge skyddande immunitet i en musmodell. I dessa studier försöker vi förbättra effekten ytterligare genom att inkorporera fusionsproteinet i polysackarid-respektive liposom-nanopartiklar. Denna doktorsavhandling visar på lovande resultat med dessa kombinationer och ger gott hopp om att vi skall kunna utveckla ett brett skyddande mukosalt influensavaccin, som kan ges till både vuxna och små barn.

Nyckelord: Mukosala vaccination; Influensa A-virus; CTA1-DD; Nanopartikel; Riktade adjuvans; Nasal immunisering; Neonatalvaccin; Universellt vaccin.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. "Porous nanoparticles with self-adjuvanted M2e-fusion protein and recombinant hemagglutinin provide strong and broadly protective immunity against influenza virus infections". Valentina Bernasconi, Beatrice Bernocchi, Liang Ye, Minh Quan Lê, Ajibola Omokanye, Rodolphe Carpentier, Karin Schön, Xavier Saelens, Peter Staeheli, Didier Betbeder, Nils Lycke. *Frontiers in Immunology, 12 September 2018* https://doi.org/10.3389/fimmu.2018.02060 *

II. "A novel combined vaccine consisting of an enzymatically active fusion protein adjuvant and lipid nanoparticles provides broadly protective immunity against influenza infection". Valentina Bernasconi, Karin Norling, Sabina Burazerovic, Karin Schön, Anneli Strömberg, Marta Bally, Fredrik Höök, Nils Lycke *(manuscript)*

III. "Targeting follicular dendritic cells with CTA1-DD adjuvant effectively promotes immune responses in neonatal mice and recovery from influenza infection". Sophie Schussek, Valentina Bernasconi, Anneli Strömberg, Karin Schön, Nils Lycke *(manuscript)*

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CONTENT

ABBREVIATIONS

Aa	Amino acid
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
AMP	Antibacterial peptides
APC	Antigen presenting cells
BAL	Broncheolaveolar lavage
BALT	Bronchus-associated lymphoid tissues
BCG	Bacillus Calmette–Guérin
BCR	B cell receptor
C	Contact mice
CCR7	Chemokine receptor type 7
cDC	Conventional dendritic cell
CDR	Complementary determining region
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CMP	Common myeloid progenitor
CpG	Cytosine-phosphate-guanine
CR	Complement receptor
cRNA	Complementary RNA
Cryo-TEM	Cryogenic transmission electron microscopy
CSR	Class switch recombination
CT	Cholera toxin
CTA	Cholera toxin A-subunit
CTB	Cholera toxin B-subunit
CTL	Cytotoxic T cells
CX3CR1	CX3C-chemokine receptor 1
DC	Dendritic cell
DPPG	Dipalmitoylphosphatidylglycerol
EEA-1	Early endosomal antigen 1
FAE	Follicle associated epithelium
FcR	Fc receptor
FcγRI	Fc-gamma receptor 1

FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FPEα	CTA1-3Eα-DD
FPEα:LNP	Liposome formulation with CTA1-3Eα-DD
FPEα:LNP+PEG	PEGylated liposome formulation with CTA1-3Eα-DD
FPEα:NPL	Porous maltodextrin nanoparticle formulation with CTA1-3Eα-DD
FPM2e	CTA1-3M2e-DD
FPM2e:LNP	Liposome formulation with CTA1-3M2e-DD
FPM2e:LNP+PEG	PEGylated liposome formulation with CTA1-3M2e-DD
FPM2e:NPL	Porous maltodextrin nanoparticle formulation with CTA1-3M2e-DD
GALT	Gut-associated lymphoid tissue
GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTMA	Glycidyltrimethylammonium chloride
HA	Hemagglutinin
HBV	Hepatitis B virus
HSC	Haematopoietic stem cells
I	Index mice
i.n.	Intranasal
IC	Immune complexes
IFN	Interferon
IgV	Immunoglobulin variable region
Ii	Invariant chain
ILF	Isolated lymphoid follicles
IRF	Interferon regulatory factor
ISCOM	Immune stimulating complexes
ISG	interferon stimulated gene
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAIV	Live-attenuated influenza vaccine
LAMP-1	Lysosomal-associated membrane protein 1
LNP	Lipidic nanoparticle/Liposome
LP	Lamina propia
LPS	Lipopolysaccharide
LT	*Escherichia coli* labile toxin

M cell	Micro fold epithelial cell
M1	Matrix protein 1
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
M-CSF	Macrophage colony stimulating factor
MDCK	Madin-Darby canine kidney cells
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
mLN	Mediastinal lymph nodes
MMR	Macrophage-mannose receptor
mo-DC	monocyte-derived dendritic cell
MPL	Monophosphoryl lipid A
mRNA	Messenger RNA
MUC	Mucins
NA	Neuraminidase
NALT	Nasopharyngeal-associated lymphoid tissues
NK	Natural killer cells
NOS2	Nitric oxide synthase 2
NP	Nucleoprotein
NPL	Porous maltodextrin nanoparticle
NTA	Nanoparticle tracking analysis
OVA	Ovalbumin
p.o.	Per oral
PA	phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PAM	Pulmonary alveolar macrophage
PAMP	Pathogen associated molecular patterns
PB1	Polymerase protein 1
PC	phosphatidylcholine
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed cell death ligand 1
PE	phosphatidylethanolamine
PEG	Polyethyleneglycol
PEMCC	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimido-methyl) cyclohexane-carbox-amide]
PEPEGMCC	1,2-distearoyl-sn-glycero-3-phosphoethanolamine -N-[maleimide(polyethy-leneglycol)-2000]

PG	phosphatidylglycerol
PI	phosphatidylinositol
pIgR	Polymeric Ig receptor
PLG	Poly(d,l-lactide-co-glycolide)
PLGA	Poly(d,l-lactic-coglycolic acid)
pMHC-II	peptide-MHC complex
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PP	Peyer´s patches
PRR	Pattern recognition receptors
PS	phosphatidylserine
REase	Restriction endonuclease
RNP	Ribonucleoprotein
s.c.	Subcutaneous
SAA	Serum amyloid A
SHM	Somatic hyper mutation
sIgA	Secretory IgA
TCM	Central-memory T cell
TCR	T cell receptor
TDM	Trehalose 6,6'-dimycolate
TdT	Terminal deoxynucleotidyl transferase
TEM	Effector-memory T cell
Tfh	T follicular helper cells
Th	T helper cell
TIV	Trivalent influenza vaccine
TLR	Toll-like receptors
TNFα	tumor necrosis factor alpha
Treg	T regulatory cell
TRM	Tissue-resident memory T cells
WHO	World Health organization
WP	Work package

1 INTRODUCTION

Vaccination has been defined as one of the most successful public health interventions to date. Our modern vaccinology era started when Edward Jenner, an English general practitioner, conducted the first scientific investigation on smallpox prevention. This was in 1796.[1] During the second half of the nineteenth century an increased focus on microorganisms and empirical discoveries, relating mainly to antibodies, brought about the development of new vaccines. The French chemist Louis Pasteur, the German scientist Robert Koch, and Emil von Behring, who got the first Nobel Prize in medicine, were the giants of this early period.[2,3-4] During the twentieth century, research focused on the nature of many different infectious diseases and how those were transmitted. There was an increasing number of pathogenic organisms being discovered and classified. This made it possible to develop new vaccines. Whereas these vaccines were developed from the whole organism, it was not until later that other forms of formulation were attempted. The most recent development in vaccine design has used nanotechnology to produce effective vaccines. This trend is now recognized as "Nanovaccinology" and its popularity has increased exponentially among vaccine manufacturers.[5] Today, nanotechnology is used to facilitate setting the diagnosis of many diseases, as well as for the delivery of biologically-active compounds in disease treatment and prevention. Both prophylactic and therapeutic nanoparticle vaccines have been developed to improve antigen (Ag) delivery and processing with the purpose of enhancing immunogeniciy.[6] In the following chapters, the reader will be guided into how we developed a subcomponent vaccine against Influenza virus infection based on the nanoparticle technology. We explored the mucosal CTA1-DD adjuvant that carried a conserved peptide from the

extracellular domain of the M2-protein, CTA1-3M2e-DD. This fusion protein was formulated into nanoparticles and the combined vaccine vector was delivered via the nasal route. Prospects are that this nanoparticle-vector can be given to both adults and neonates.

1.1 The mucosal immune system and vaccination

1.1.1 Overview of the mucosal immune system

Before going into details about the vaccine development itself, it is useful to keep in mind that the goal of this project was to deliver an effective mucosal vaccine. It is, therefore, helpful at this point to start with an introduction of how the immune system is organized and works at the mucosal membranes. The mucosal immune system is in fact the largest lymphoid organ in the human body, with a surface area close to 400 m^2 in humans, and it comprises the mucosal membranes of the small and large intestine, and the urogenital and respiratory tracts (Figure 1).

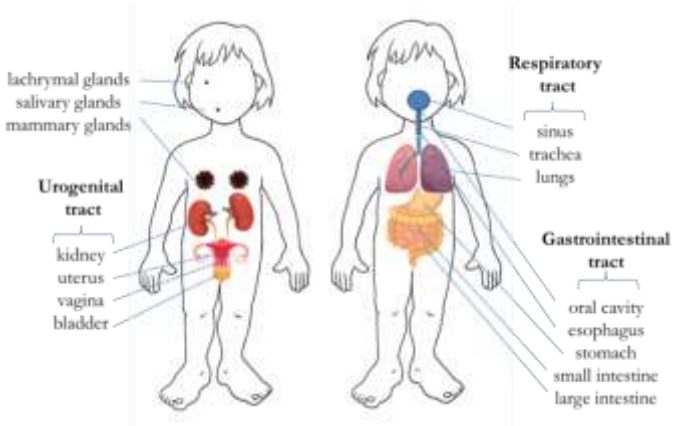


Figure 1. Mucosal tissues in the human body. Mucosal immune system includes the mucosal membranes of the gastrointestinal, respiratory and urogenital tracts.

Within the mucosal immune system, anatomically distinct compartments can be found, separated into inductive and effector sites based on their anatomical and functional properties. Inductive sites play a major role in Ag sampling and the stimulation of adaptive immune responses; the effector sites are where immune protection against, for example, a pathogen is carried out (Figure 2). The **inductive sites** are collectively called mucosa-associated lymphoid tissue (MALT) and include gut-associated lymphoid tissue (GALT), which includes the Peyer´s patches (PP) and the isolated lymphoid follicles (ILF) of the small intestine, the colon patches and the appendix. Separated from the GALT there are the mesenteric lymph nodes (MLN). To the MALT we also count the nasopharyngeal-associated lymphoid tissues (NALT) and bronchus-associated lymphoid tissues (BALT), which drain to the mediastinal lymph node (mLN), and organized lymphoid tissues in the genitourinary tract. The **effector sites** include the mammary and salivary glands and the non-organized lamina propia (LP) of the small intestine and of the respiratory and genitourinary tracts. The LP is the layer of connective tissue between the epithelium and the muscularis mucosa, which hosts smooth muscle cells, fibroblasts, and lymphatic and blood vessels. The LP is the main site for effector lymphocytes and where we find most of the Ag-specific IgA producing plasma cells and resident T cells.[7,8,9]

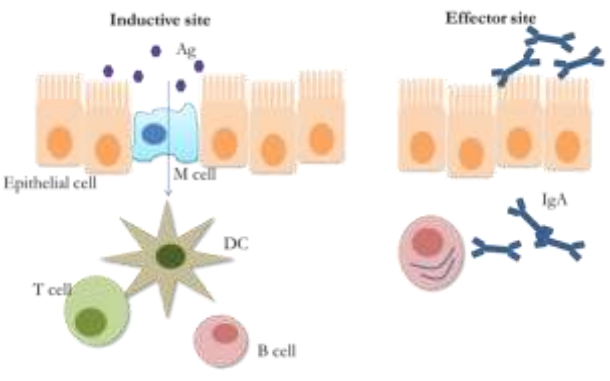


Figure 2. Inductive and effector sites at the mucosal surface. M cells are the specialized epithelial cells that transport Ags from the lumen to cells of the immune system. At the effector sites, T cells and plasma cells producing dimeric IgA dominate.

The mucosal surfaces are thin and permeable barriers to allow for their physiological functions, i.e. gas exchange (the lungs), food absorption (the gut), sensory activities (eyes, nose, mouth, and throat), and reproduction (uterus and vagina). This results in that mucosal membranes are at risk and vulnerable to infections. At the mucosal sites the first line of defense against pathogens is an anatomic barrier, which mainly consists of an intact single cell epithelial layer, above which a mucus layer is useful to protect the body from the entrance of microorganisms.[10] However, in the respiratory and genitourinary tracts the epithelial layer is more complex and in places pseudostratified or squamous. In the oral cavity, pharynx, esophagus, urethra and vagina there is a multilayered epithelial lining. The mucus and cilia effectively trap microorganisms, which helps expel them from the body.[11,12]

Additional mechanisms may inhibit the growth of microbes, such as the acidic pH of the stomach or molecules secreted at the mucosal surface, such as antibacterial peptides (AMP), interferons (IFN), or collectins. The term antimicrobial peptide traditionally refers to small (<100 amino acids) cationic peptides that have antimicrobial activity and exert substantial immunomodulatory influence locally by inducing secretion of cytokines, recruiting immune cells or participate in the remodeling of injured epithelia.[13] Examples of AMPs are lysozyme and lactoferrin. Lysozymes are hydrolytic enzymes found in tears and mucus secretions that can cleave the peptidoglycan layer of bacterial cell walls.[14] Lactoferrin is a globular glycoprotein able to sequester free iron and, thereby, removing an essential substrate required for bacterial growth. Moreover, lactoferrin binds to the lipopolysaccharide of bacterial walls, affecting the membrane permeability and resulting in cell lysis.[15] INFs are secreted proteins that are commonly grouped into three types. Type I IFNs are also known as viral IFNs and include IFN-α (leukocytes) and IFN-β (fibroblasts). Type II IFN is also known as gamma IFN (IFN-γ). The more recently

described type III IFN is IFN-λ, which has been found not only to protect against virus infection, but it is also a potent immunomodulatory of mucosal immune responses. Type I IFNs are induced by virus infection, whereas IFN-γ is induced by antigenic and inflammatory stimuli. Most types of virally infected cells are capable of synthesizing IFN-α/β in cell culture. By contrast, IFN-γ is synthesized only by certain cells of the immune system, including natural killer (NK) cells, $CD4^+$ Th1 cells, and $CD8^+$ T cells. The IFNs exert their effects through cell surface receptors, which initiate a signaling cascade (JAK-STAT pathway) that eventually affects the transcription of different genes.[16] Collectins are surfactant proteins that are present in serum, lung secretions, and mucosal secretions that can directly kill some pathogens by disrupting their lipid membranes or indirectly by enhancing their phagocytosis.[17,18,19]

1.1.2 The mucosal immune system of the respiratory tract

Understanding the mucosal immune system of the airways is important for the development of vaccines against lung infections, such as RSV or Influenza virus. Because the vaccine developed in this research project is directed to protect against influenza virus infection of the respiratory tract, we have focused on intranasal vaccine administration.

The respiratory tract is the body's second-largest mucosal surface area after the digestive tract.[20] It is divided into upper (from the nasal and oral cavities to the throat) and lower (trachea and lung) respiratory tracts. While the upper respiratory tract is exposed to inhaled air and, thus, at risk for pathogens (Influenza virus) or opportunistic microorganisms (*Haemophilus influenzae* and *Streptococcus pneumoniae*) or even resident microflora, the lower respiratory tract is essentially sterile in healthy individuals.[21] The luminal side of the respiratory tract is generally physically separated by a single epithelial cell layer with tight junctions between the cells. A more complex pseudostratified or squamous

epithelium is found in the oral cavity, inferior part of the pharynx and esophagus to protect from abrasions. Alveoli are characterized by a very thin simple epithelium layer which favors the exchange of gases (Figure 3). The underlying LP contains elastin, which plays a role in the elastic recoil of the trachea to facilitate breathing. Superficial blood vessels secure warming of the inhaled air. The epithelial cells have well-developed cilia and produce mucus composed of mucins (MUC), which are high molecular weight, heavily glycosylated protein (glycoconjugates).[22] At the bronchi, mucus and movement of cilia prevent large foreign bodies (>5 mm) from drifting into the alveoli (mucociliary clearance). However, pathogens have developed ways to evade the physical barrier and can, therefore, establish infections in the alveoli of the lungs.[10]

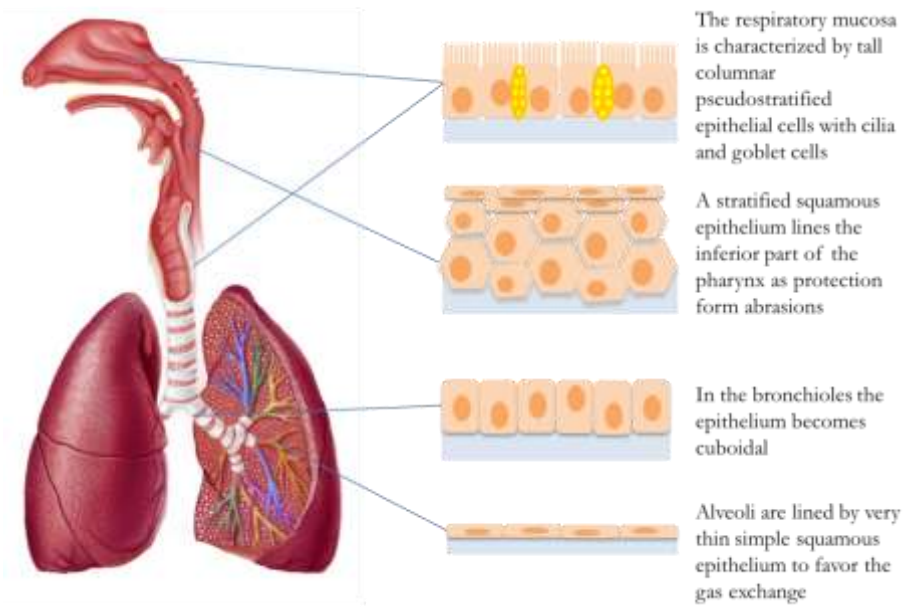


Figure 3. The structure of the respiratory epithelium at different sites in the respiratory tract. The respiratory epithelium is generally characterized by a single epithelial cell layer. A more complex pseudostratified or squamous epithelium is found in the oral cavity, inferior part of the pharynx and esophagus. Alveoli are characterized by a very thin simple epithelium layer which favors the exchange of gases.

1.1.3 Innate and adaptive immune responses

The innate and the adaptive immune systems continually interact with each other to provide an effective immune response.[8] The **innate immune** system provides a first-line of defense against pathogenic agents. However, these responses are not specific to a particular pathogen, but rather respond to microbial signals that are shared by many microorganisms. By contrast, the actions of the **adaptive immune** system are very specific and dictated by the receptor mediated recognition of a unique epitope associated with a specific microorganism. At the first encounter with a pathogen the adaptive immune response takes longer to develop, but upon re-exposure to the pathogen it will respond rapidly.[9] Thus, in contrast to the innate immune system, the actions of the adaptive immune system are specific to a particular pathogen. The two arms of the adaptive immune system are B cells and T cells, which express distinct receptors for the Ag.[9]

The protective defenses of the innate immune system at the mucosal sites include pattern recognition receptors (PRRs), which are found on the cell membrane or intracellularly of innate immune cells.[23] The most important innate receptors are the toll-like receptors (TLRs), but other examples are pulmonary surfactant protein, and C-reactive protein.[24,25,26] The PRRs recognize pathogen associated molecular patterns (PAMPs) like LPS (endotoxin), peptidoglycan (cell walls), lipoproteins (bacterial capsules), hypo methylated DNA (CpG found in bacteria and parasites), double-stranded DNA (viruses), and flagellin (bacterial flagella), produced by microbial cells.[27,28,29] Once PAMPs are recognized by PRRs, the complement proteins are activated and opsonization, cytokine release, and phagocytosis are induced.[29] There are many cell types that belong to the innate immune system, such as dendritic cells (DC) macrophages, monocytes and granulocytes. While macrophages and DCs are located in the tissues, monocytes are blood circulating cells that can differentiate

into specific tissue macrophages or DCs.[30] DCs are important in Ag-presentation and activation of $CD4^+$T cells.[31] Granulocytes include neutrophils, eosinophils, and basophils/mast cells. Neutrophils are highly active phagocytic cells and generally arrive first at a site of inflammation. Eosinophils are important in resistance to parasites and for maintaining niches of long-lived plasma cells and memory T cells. Basophils in the blood and mast cells in the tissues release histamine and other substances and are involved in the development of allergies.[32,33,34] I will focus more on DCs in the following chapters.

B cell or **humoral immunity** develops against antigenic epitopes that most often are molecules associated with extracellular pathogens.[35,36] B cells are produced in the bone marrow and then travel to the lymph nodes where they await Ag exposure.[37] When exposed to foreign Ags, naïve B cells undergo T cell-dependent or T cell-independent activation. The latter occurs only against certain Ags with repetitive units, such as lipopolysaccharides, dextran or bacterial polymeric flagellin. Antibodies generated in this way tend to have lower affinity, do not drive memory development and are mostly IgM antibodies.[38]

By contrast, most antibody responses are against T cell-dependent Ags, which involves help from $CD4^+$ T cells to develop antibody responses. Hence, the B cells need additional helper factors to undergo cell division and differentiate into plasma cells for antibody production at effector sites. The B cell receptor (BCR) binds specifically to the Ag, which most often hosts a complex epitope that is non-linear and with a 3D-structure. Subsequent to the BCR-recognition of the Ag-epitope, the Ag is taken up through receptor-mediated endocytosis and degraded to peptides. These peptides can then be presented by MHC II molecules to stimulate cognate interactions with $CD4^+$ T cells in the lymph node. This initiates the process which leads to the development of helper T cell functions.[39] B cell proliferation, immunoglobulin class switch recombination (CSR),

and somatic hyper mutation (SHM) primarily occur in the germinal center (GC), which is a hallmark of T cell-dependent responses and allows for a strong and effective antibody response.[36] This results in a much better immune response and the development of memory B cells and long-lived plasma cells.[40]

There are five classes (isotypes) of antibody molecules, IgG, IgM, IgA, IgE and IgD, which have different and complementary functions (Figure 4). Each isotype has a different heavy chain. This is true in mice, as well as in humans. IgM is the first antibody produced by B cells challenged with Ags and it is expressed on the surface of B cells as monomer. It is the B cell receptor (BCR) for Ag recognition in both naïve and in some memory B cells. IgM is a strong complement activator and often has better neutralizing ability than IgG. IgG is expressed on the surface of Ag-activated B cells and it is the most prevalent Ig-class in serum and in extravascular spaces. Mouse IgG isotypes displayed marked differences in bactericidal (IgG3 >> IgG2b > IgG2a >> IgG1) and opsono-phagocytic (IgG3 > IgG2b = IgG2a >> IgG1) activity. The IgG2a has been found to correlate with survival and protection against influenza virus challenge. The IgD antibody is co-expressed on naïve B cells together with IgM and plays a role in B cell development. It is found in very low concentrations in serum. IgE is the antibody involved in allergic reactions and parasitic infections.

IgA is the primary isotype induced and secreted at mucosal sites. IgA is resistant to digestion and can activate the complement pathway when aggregated.[41,42,43] The IgA is produced as a dimer and linked together with a J-chain by the plasma cell. After secretion, mucosal IgA is transported from the basolateral epithelial compartment to the apical/luminal side.[44] Transport of IgA to the lumen is mediated by the polymeric Ig receptor (pIgR), which is expressed at the basolateral side of the epithelial cells that line the mucosal surfaces.[45] During this transport, the pIgR is

proteolytically cleaved and the extracellular portion of the molecule, the secretory component, is released in association with the pIgA, forming altogether the secretory IgA (sIgA).[46] These antibodies are secreted in milk, tears, saliva and are found in the mucus in the respiratory, genital- and gastrointestinal tracts. Monomeric IgA is found in serum, but at much lower concentrations than IgG.

At the luminal site, pIgA binds specifically to Ags in the mucus. In addition IgA can bind Ags prior to the transcytosis and form immune complex, which are then discarded into the luminal excretory pathway.[47] A few studies have also documented that during the transport through epithelial cells, SIgA could neutralize virus infections.[48]

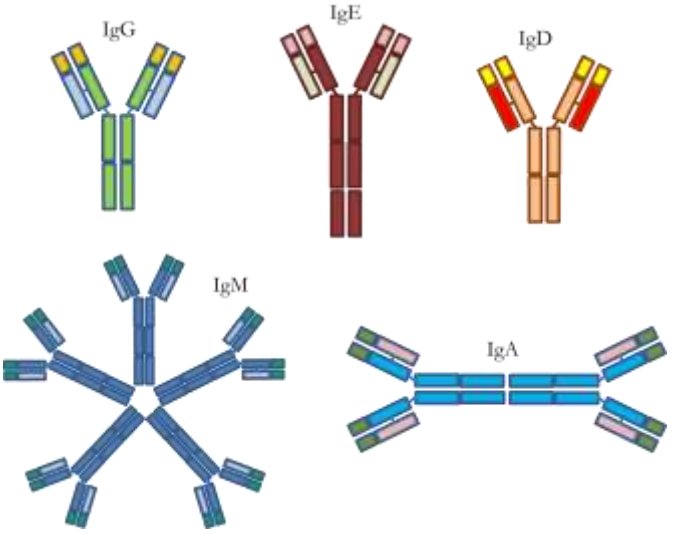


Figure 4. The different immunoglobulin classes. IgG, IgE and IgD are monomers. IgM can be found in monomeric or pentameric forms; the monomer form is the B cell receptor that recognizes Ag; the pentameric form is the first antibody released during the primary immune response. IgA can be monomeric (serum) or dimeric (sIgA in secretions at mucosal membranes).

Cell-mediated immunity is effective at eliminating intracellular pathogens. T cells are formed in the bone marrow, but critically undergo selection and additional maturation to become functional $CD4^+$ and $CD8^+$ T cells in the thymus.

$CD4^+$ cells (or T-helper cells) have the CD4 co-receptor and their T cell receptor (TCR) can only recognize peptide epitopes expressed together with MHC II protein on antigen presenting cells (APC). $CD4^+$ T cells are essential for antibody-mediated immunity and in helping $CD8^+$ T cell expansion and maturation. They also exert regulatory functions or participate as effector cells to control infecting pathogens. During TCR activation in a particular cytokine milieu, naive $CD4^+$ T cells may differentiate into one of several functional subsets of Th cells, including Th1, Th2, Th17, Tfh and Treg, as defined by their pattern of cytokine production and function.[49] Th1 and Th2 can be distinguished mainly by the cytokines produced by the cells, but also through the expression of different patterns of cell surface molecules. Th1 cells promote cell-mediated immunity against viruses and intracellular bacteria and Th2 are involved in antibody-mediated immunity against parasites as well as play a major role in allergic conditions, such as asthma. With regard to cytokine expression, Th1 cells make IFN-γ as their signature cytokine and also uniquely produce lymphotoxin. Th1 cells tend to be good IL-2 producers, and many make TNF-α as well. By contrast, Th2 cells fail to produce IFN-γ or lymphotoxin. Their signature cytokines are IL-4, IL-5, and IL-13. They also make TNF-α, and some produce IL-9. Although initially thought to be unable to make IL-2, later results indicated that Th2 cells could often produce relatively modest amounts of IL-2. Th17 cells are characterized by the production of IL-17A, IL-17F, IL-21 and IL-22 as signature cytokines, molecules not produced by Th1 or Th2 cells. They help fight against extracellular bacteria and fungi. Tregs are regulatory T cells and produce IL-10 and TGF-β. Finally, the subset that is involved in

regulating the B cell response in the GC is named T follicular helper cells (Tfh). These cells produce the cytokines required for class-switching, i.e. IL-4, TGF-β and IFN-γ as well as cytokines necessary for differentiation and maturation into memory B cells and long-lived plasma cells, such as IL-21 (Figure 5).[49]

$CD8^+$ T cells (or cytotoxic T cells) have the CD8 co-receptor and only recognize peptide plus MHC I complexes. All nucleated body cells, except for mature erythrocytes, express the MHC I molecule on their surfaces. $CD8^+$ T cells are essential for cell-mediated immunity and protecting against intracellular pathogens. These cells produce IFN-γ, TNF-α, perforin and granzyme among other factors (Figure 5).[50]

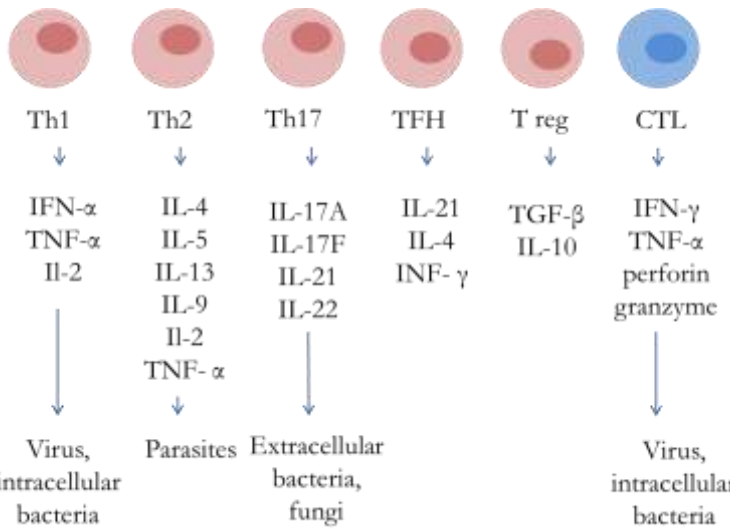


Figure 5. T cell cytokine production. Upon differentiation, T cells produce cytokines, which feed back into the cellular milieu, amplifying and balancing the immune response to promote specific pathogen clearance and host survival.

Activated T-cells undergo clonal expansion and differentiation into the functional subtypes mentioned above and some develop into memory T-

cells for future protection against infection with a specific pathogen.[51,52,53] A T cell response typically peaks 7–15 days after initial Ag stimulation. Over the next few days, 90–95% of Ag-specific T cells then die off, leaving behind a pool of memory cells with a range of phenotypes and functionalities. For both $CD4^+$ and $CD8^+$ T cells, there are three main subclasses of memory cells: central-memory (TCM), effector-memory (TEM) and tissue resident memory T cells (TRM). TCM cells are commonly defined phenotypically as expressing high levels of the IL-7 receptor (CD127), high levels of adhesion markers like CD44 and CD62L, low levels of the surface marker killer cell lectin-like receptor subfamily G member 1 (KLRG-1), and high levels of the C-C chemokine receptor type 7 (CCR7). Furthermore, TCM cells are functionally characterized by their increased potential for proliferation after Ag reencounter. TEM cells express low levels of CD62L, low levels of CD127, high levels of KLRG-1, and are deficient in CCR7. TEM cells display rapid effector function (granzyme B and IFN-γ production), but a limited proliferative potential. The high expression of CD62L and CCR7 by TCM cells allow for preferential homing to the secondary lymphoid organs, which constitutively produce the CCR7 ligands CCL19 and CCL21. TEM, since they lack CCR7 and CD62L expression, circulate through non-lymphoid tissues and are the "first responders" at the peripheral site where reinfection could occur.[54] Once formed, subsets of memory cells can survive for decades (the half-life of memory T cells is 8–15 years), providing protection for the better part of a lifetime.[55] The TRM population expresses CD69 and CD44 ($CD8^+$ T cells also express CD103) and are located in the tissues, where they reside for long periods of time. The function of these $CD4^+$ and $CD8^+$ memory T cells is only beginning to be investigated, but several model systems have revealed an absolute critical function for protection against infection. For example, protection against influenza virus has been found completely dependent on the lung resident memory $CD4^+$ and $CD8^+$ T cell populations.[56]

1.1.4 Antigen uptake and presentation for stimulation of adaptive immune responses

In this research I have given a special focus to DCs, which are central to priming of immune response against T cell dependent Ags. DCs reside in most peripheral tissues (skin and mucosae), where they represent 1%–2% of the total cell numbers.[57,58] The DCs constitutively patrol our tissues and, when activated, migrate to the draining lymph nodes to present Ag to the T cells. At the mucosal membrane, DCs can take up Ags directly from the lumen or they can interact with macrophages ($CXCR1^+$ cells) taking up Ags from those cells. Macrophages are distinguished as larger vacuolar cells that effectively clear the tissues from apoptotic cells, cellular debris and pathogens. These cells are phenotypically defined as $F4/80^{high}$ cells in mice. By contrast, DCs are usually defined as cells with a stellate morphology that can efficiently present Ags on MHC molecules and activate naive T cells. In mice, DCs are defined as $CD11c^{high}$, MHC II^+ cells. DCs generally display a short half-life of approximately 3–6 days in the tissues and are constantly replenished from bone marrow precursors in a strictly Flt3L-dependent manner.[59] The DCs are located in the mucosal membranes to secure that foreign Ags can be captured and, if recognized by T cells, will initiate an immune response. Activated DCs migrate to the draining lymph nodes and more specifically to the T cell zone, where the CCR7-ligands CCL19 and CCL21 are expressed.[60] These DCs are called migratory DCs, while the DC residing in the lymph nodes are named resident DCs.[61] Mice deficient in CCR7 signals show severe defects in lymphoid tissue architecture and immune responses. These defects are due to impaired migration of $CCR7^+$ DC and $CCR7^+$ T cells into the T cell zones of secondary lymphoid organs and altered DC maturation.[62] A chemokine receptor, CCR6, has been shown to control the migration of immune cells toward the mucosal surfaces.[63] The ligand for CCR6 is the chemokine CCL20 and it is expressed by a variety of

epithelial cell types including keratinocytes, pulmonary epithelial cells, and intestinal epithelial cells.[64]

DCs are a heterogeneous cell population divided into many different subsets on the basis of the expression of some of the following surface markers: CD103 (also known as integrin αE), CD11b (also known as integrin αM), CX3C-chemokine receptor 1 (CX3CR1), F4/80, CD8α, CD24, CD172a (also known as SIRPα and SHPS1), XC-chemokine receptor 1 (XCR1), Clec9A (also known as DNGR1), E-cadherin (also known as cadherin 1) and CD64 (also known as FcγRI). In mice three main lineages of DCs can be found: conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte-derived DCs (mo-DCs).

cDCs are specialized in Ag processing and can efficiently present endogenous and exogenous Ag. cDCs are differentiated into cDC1 which are $CD8\alpha^{+}CD103^{+}$ $CD11b^{-}$ DCs, and cDC2 which are $CD11b^{+}CD172a^{+}CD64^{-}$ DCs.[65-67,68] Whereas DC2 present peptides to $CD4^{+}$ T cells, the DC1 are also able to cross-present peptides to $CD8^{+}$ T cells, which recognize peptides in the context of MHC I. Hematopoietic stem cells (HSC) give rise to DC and monocyte-derived cells by distinct routes marked by differences in the relative expression of interferon regulatory factor 8 (IRF8) and IRF4. Monocytes are IRF4/8 low but can be induced to differentiate into mo-DC (Figure 6).

pDCs are found in the blood and in many tissues and have a highly developed secretory compartment.[69] They sense viral and bacterial pathogens and a hallmark is that they produce large amounts of type I interferons in response to infections.[70] pDCs have been found to contribute to inflammatory responses and cause pathology by driving a pro-inflammatory response in the tissue.[71]

moDCs originate from monocyte infiltrates as a consequence of inflammation in lymphoid and non-lymphoid organs. moDCs are also called DC3 and are $CD11b^{+}CD64^{+}$.[72] These cells are phenotypically difficult to discern from cDCs because they share similar expression patterns of MHC-II, CD11b, and CD11c. However, some markers differ and, for example, they express the Fc-gamma receptor 1 (FcγRI).[73,74]

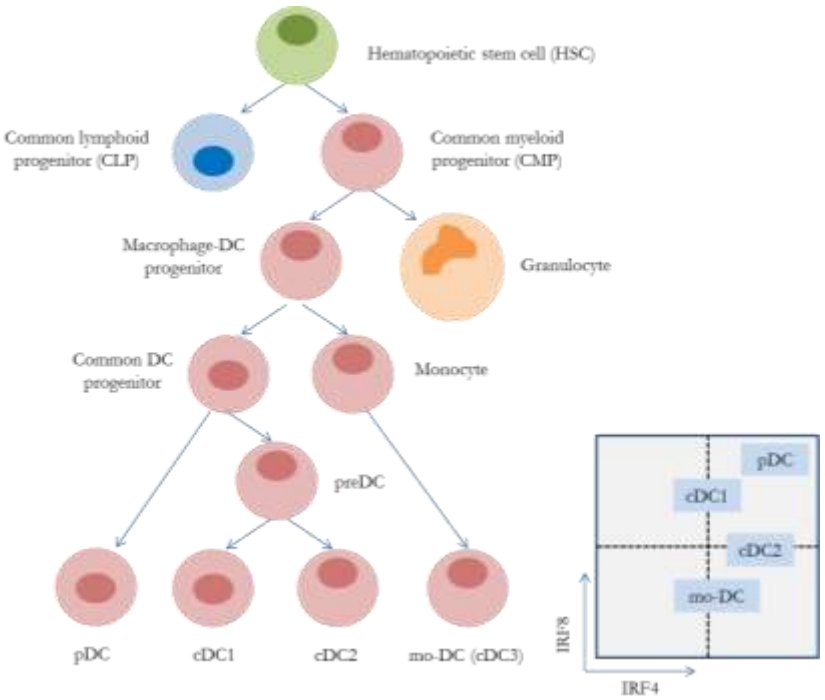


Figure 6. Model of hematopoiesis. Common lymphoid and common myeloid progenitors (CLP,CMP) arise from the HSC. DCs arise from a macrophage-DC progenitor. HSC give rise to DC and monocyte-derived cells by distinct routes marked by differences in the relative expression of IRF8 and IRF4 as shown in schematic bivariate plot.

Antigen uptake by DCs

In areas with a specialized follicle associated epithelium (FAE), specialized micro fold (M) epithelial cells take up Ags from the lumen and

hand it over to the sub epithelial DCs. Such cells are found in the PP in the intestine and in the NALT of the airways.[75,76,32,33,34] Tissue DCs are able to capture particles as well as soluble molecules. This includes pathogens, infected cells, apoptotic cells, or substances from these cells. The DCs can use several different and complementary pathways for Ag acquisition. The **receptor-mediated endocytosis** pathway allows for the uptake of macromolecules through specialized regions of the plasma membrane, termed coated pits. Particulate and soluble Ag can also be internalized by **phagocytosis** or **macropinocytosis** (Figure 7).[77]

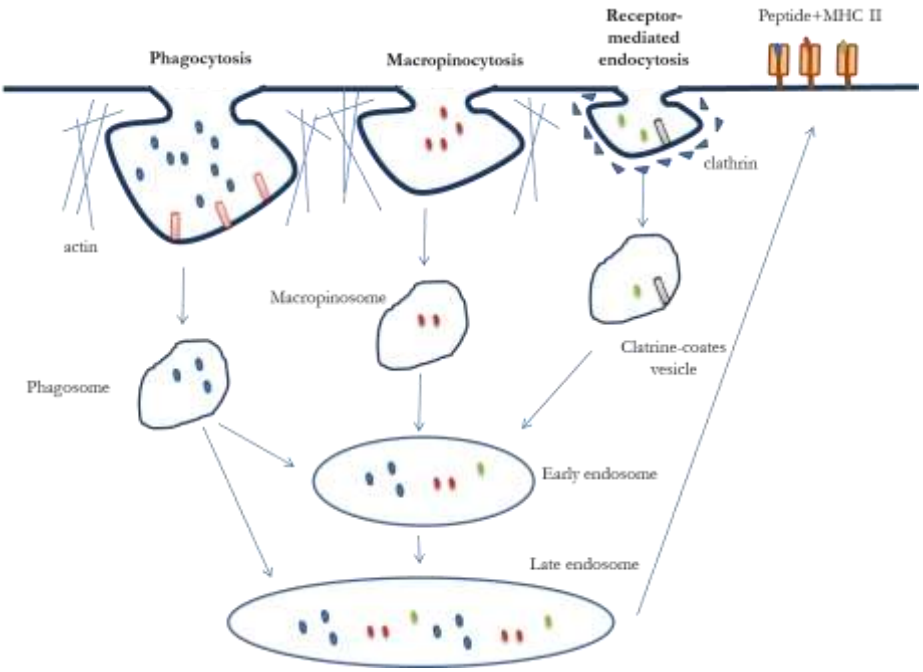


Figure 7. Pathways of exogenous Ag uptake in DCs. DCs internalize extracellular Ags using three main endocytic pathways. Phagocytosis is an endocytic process in which opsonized particles bind to specific receptors on the DC surface and enter cells in membrane-derived phagosomes. Macropinocytosis mediates non-specific uptake of soluble Ags into the cell via macropinosomes. Receptor-mediated endocytosis is a process in which small soluble Ags bind to specific receptors on the DC surface which internalizes in clathrin-coated vesicles.

Receptor-mediated endocytosis is initiated by a signal in the cytoplasmic tail of the endocytic receptor, which is recognized by a family of adaptors proteins responsible for the recruitment of clathrin lattices and for the formation of clathrin-coated endocytic vesicles.[78] Furthermore, there is a large number of endocytic receptors that are selectively expressed by subpopulations of immature DCs. For example, mouse immature DCs express receptors for the Fc portion of immunoglobulins (FcR) such as FcRI (CD64), FcRII (CD32) and FcRIII (CD16).[79,80] Immature DCs also express complement receptor CR3 and CR4.[81] Moreover, heat shock proteins (HSPs) derived from tumor cells or infected cells are internalized through specific membrane receptors.[82] These are called scavenger receptors (SRs) and are cell surface glycoproteins defined by their potential to chemically bind modified low-density lipoproteins. SRs are implicated in internalization of various bacteria.[83] DCs express also several transmembrane C-type lectins which bind ligands in a Ca-dependent manner, including the macrophage-mannose receptor (MMR), expressed on alveolar and differentiated macrophages, blood and monocyte-derived DCs, interstitial DCs in dermis of the skin and on the thymic epithelial cells.[84,85] Following uncoating of clathrin, Ags contained in clathrin-coated vesicles are delivered to early endosomes and eventually to Ag processing compartments for proteolytic degradation and peptide-MHC II (pMHC-II) formation. After formation in the endosomal Ag processing compartments, pMHC-II complexes traffic to the plasma membrane to allow for TCR-recognition and activation of $CD4^+$ T cells.

Phagocytosis and macropinocytosis are actin dependent, require membrane ruffling, and result in the formation of large intracellular vacuoles. Phagocytosis is generally receptor mediated, whereas macropinocytosis is a cytoskeleton-dependent type of fluid-phase endocytosis. Phagocytosed Ags can fuse with $MHC\text{-}II^+$ lysosomes to

generate phagolysosomes or they can be directly targeted into late endosomal/lysosomal Ag processing compartments to generate pMHC-II complexes. Macropinocytosis allows DCs to rapidly and nonspecifically sample large amounts of material in the surrounding fluid.[84] Ags in macropinosomes are transferred into early endosomes that eventually fuse with multivesicular late endosomal/lysosomal Ag processing compartments. Macropinocytosed Ags are degraded and loaded on MHC-II in these compartments.

Efficient Ag internalization is a specific attribute of immature DCs. Noteworthy, during maturation, DCs downregulate their endocytic capacity, thus, limiting the ability to take up new Ags as they leave the peripheral tissues. This down-modulation of the Ag-internalization machinery occurs through two independent mechanisms: a decrease in cell surface receptor expression (e.g., MMR/FcR) as well as the down-modulation of both macropinocytosis and phagocytosis.[84]

Whereas some studies on Ag processing have mainly investigated the degradation of soluble proteins or bacterial or viral Ags, few other studies have analyzed the processing of nanoparticle-associated Ags. Rincon-Restrepo et al. recently demonstrated that a differential activation of T cell immunity could be achieved through differential modes of intracellular trafficking of nanoparticles hosting certain features. These investigators engineered two different nanoparticle systems in which Ag was either encapsulated within the core or decorated onto the surface of the nanoparticles. Ags encapsulated within the core of the particle were better at driving cytotoxic $CD8^+$ T cell responses, while Ags on the surface of nanoparticles preferentially augmented $CD4^+$ T cell and antibody responses.[86] Hence, intracellular trafficking and localization predominantly to endosomal or lysosomal compartments may be used to tailor specific immune responses. Therefore, nanocarriers design can have

a critical impact on the quality of the induced adaptive immune response.[87]

Antigen presentation and DCs maturation

APCs take up Ag and degrade proteins into peptides that are bound to MHC molecules, which can be recognized by the TCR. The MHC is a highly polymorphic set of genes that encode for molecules essential to self/non-self-discrimination and Ag processing and presentation. There are two classes of MHC molecules, **MHC-I** and **MHC-II**, and are both transmembrane glycoproteins belonging to the immunoglobulin supergene family.

MHC I molecules are found on the cell surface of all nucleated cells in vertebrates. These molecules are composed of a transmembrane alpha chain associated non-covalently with the β2-Microglobulin chain. MHC-I molecules are specialized for the presentation of peptides derived from endogenous proteins (intracellular Ags) to the TCR of $CD8^+$ T cells. Most peptides to be loaded on MHC I molecules are generated by proteasome degradation of newly synthesized ubiquitinated proteins. The resulting peptides are transferred to the ER by transporters (TAP9) and loaded on new MHC I molecules under the control of a loading complex composed of several ER resident chaperons.[88] Once peptides are bound to MHC I molecules, they are rapidly transferred through the Golgi apparatus to the plasma membrane (Figure 8).

MHC-II molecules are composed of two non-covalently linked transmembrane chains, the alpha and beta chains. MHC II molecules are found only on APCs such as DCs, mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells. They are specialized for the presentation of extracellular Ags to the TCR of $CD4^+$ T cells. Soon after synthesis in the ER, MHC II dimers associate to a trimer of

invariant (Ii) chains.[89] They exit the ER and pass through the Golgi apparatus before being transported to the endocytic pathway. The Ii chain is degraded by several proteolytic enzymes of the cathepsin family and MHC II dimers become competent to bind antigenic peptides.[90,91] Once loaded with peptides, Ii chain-free pMHC-II complexes reach the plasma membrane (Figure 8).

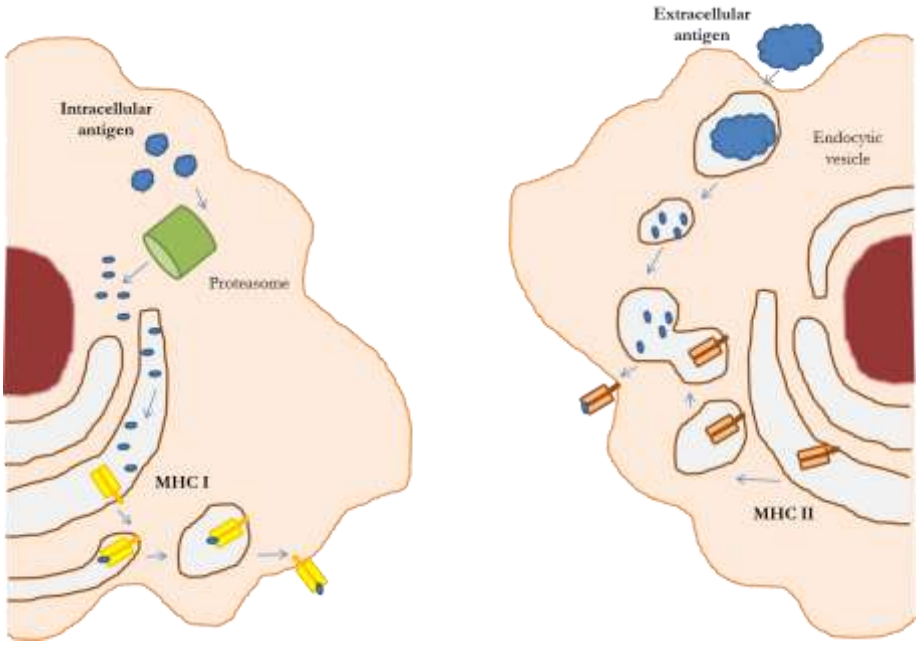


Figure 8. MHC-I and II Ag processing and presentation. MHC I molecules present peptides derived from endogenous proteins (intracellular Ags), while MHC II molecules present peptides derived from extracellular Ags. Some DCs can cross-present peptides, which means that when extracellular proteins are taken up they can be degraded and peptides presented on MHC I.

MHC I synthesis and half-life are increased upon induction of DC maturation.[92] Targeting of proteins for proteasomal degradation requires their ubiquitination. Importantly, not only endogenous Ags can be presented by MHC I molecules, but also exogenous Ags internalized by various pathways. This phenomenon is termed cross-presentation. It is

believed that phagocytosis is a major route for Ag uptake for cross-presentation, but also macropinocytosis allows for receptor-independent cross-presentation of soluble Ags to $CD8^+$ T cells.[93,94] In addition, FcR-mediated uptake of immune complexes, opsonized liposomes, or opsonized dead cells could be involved in efficient cross-presentation.[95,96]

MHC II molecules are exposed on the surface of immature DCs. However, the DCs upregulate the expression of MHC II molecules upon activation and maturation. This leads to a stronger expression of pMHC-II complexes on the surface of the DCs. Whereas Ag degradation is relatively inefficient in immature DCs, internalized Ags can remain intact in lysosomal compartments for several days. As a consequence, the availability of antigenic peptides to load on MHC II molecules could, therefore, be limited.[97] Once at the cell surface, MHC II molecules are rapidly internalized and can associate with new peptides in recycling endosomes before going back to the cell surface, or they are directed to lysosomes, where they will finally be degraded.[98,99] This pathway results in a short-term flux of peptide presentation by the immature DCs, but this is probably not sustained for long enough to prime $CD4^+$ T cells in the draining lymph nodes. On the other hand, following maturation, upregulation of MHC II synthesis and protease activity is observed in the DCs.[92,100] This leads to a dramatically increased complexes formation between available peptides and MHC II molecules. pMHC complexes are then rapidly formed and transported to endosomal vesicles, where they co-localize with costimulatory molecules before being delivered to the cell surface as clusters of molecules responsible for priming of the $CD4^+$ T cell.[97,101] Following maturation, the endocytosis activity in the DCs decreases, and additional transport of peptide and MHC II molecules going to lysosomal degradation is greatly reduced. This results in stabilization of surface expression of pMHC-II complexes.[98] Although at this stage the DCs even downregulate the synthesis of MHC II

molecules, fully mature DCs can still form new pMHC complexes by recycling MHC II molecules from the cell surface.[102] Anti-inflammatory cytokines can interfere with the regulation of MHC II processing pathways in DCs. Macrophage colony stimulating factor (M-CSF) induces a rapid and sustained upregulation of MHC II synthesis which influences the expression level of the complex in immature DCs. This occurs, however, without stabilizing these complexes on the cell surface, which negatively affects the ability to prime the $CD4^+$ T cells.[103] Also, other factors influence MHC II synthesis and expression. For example, IL-10 inhibits the rise in protease activity by increasing endosomal pH which impairs Ag processing and presentation in mature DCs.[104]

1.1.5 Priming of naive $CD4^+$ and $CD8^+$ T cells

After DCs successfully have taken up and processed the Ags into peptides, they migrate to draining lymph nodes and prime the T cells in the T cell zone. T cell priming and activation relies on three distinct signaling pathways. First, the TCR has to recognize the pMHC complex. Then, CD28 on the T cell binds to costimulatory molecules (CD40, CD80, CD86) on the mature DC. This gives a survival signal to T cell and allows it to respond to additional stimulant in the form of cytokines (IL-6, IL-12, IL-4, TGFβ) produced by the DC (Figure 9). Of note, when pMHC complexes are presented in the absence or with poor co-stimulation, the outcome is often immune tolerance.[105,106] Hence, this drives the differentiation of regulatory T cells (Tregs) that act to exert immune suppression on the T cell response. DCs can also actively silence T cell activation by expressing molecules such as programmed cell death ligand 1 (PD-L1).[107] The crosstalk between T cells and DCs is bidirectional: CD40L-expressing T cells critically promote DC maturation.

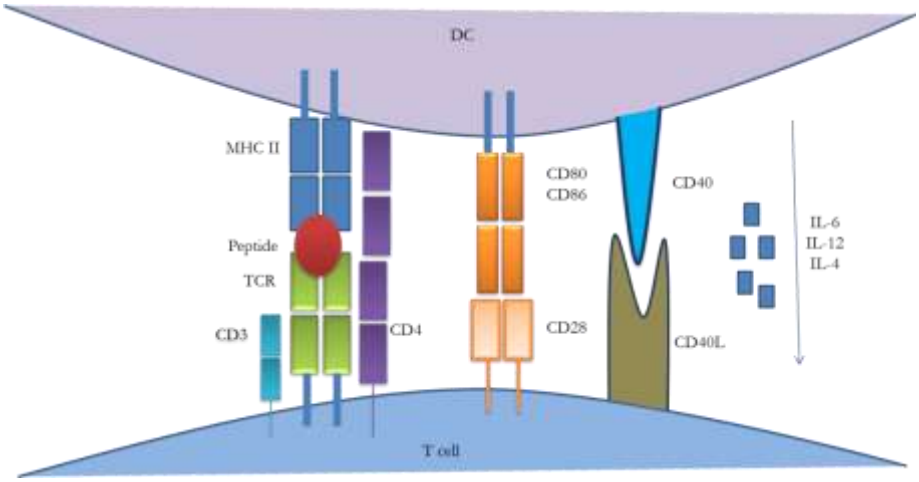


Figure 9. Signals required for naïve T cell priming by DCs. The T cell encounters a DC bearing its cognate peptide in an MHC molecule, and binds the pMHC though CD4 or CD8. Subsequently, co-stimulation occurs through DC-bound CD86, CD80 and CD40. This induces full activation of the T cells, leading to cell division and differentiation into effector functions.

The TCR recognition and concomitant costimulatory signals delivered by the APC induce a transcriptional program resulting in robust IL-2 production, which is the prime autocrine and paracrine growth factor that stimulates T cell division. When activated $CD8^+$ T cells differentiate in the presence of IFN-α/β and IL-12, they develop into cytotoxic T cells. [54] The $CD4^+$ T cell subsets are Th1, Th2, Th17, Tfh and Tregs. The production of IFN-α/β and IL-12 cytokines in response to an intracellular pathogen (viruses and mycobacterium tuberculosis) upregulate the expression of the transcription factor T-bet, which is a master gene involved in Th1 differentiation.[108] On the other hand, when activated $CD4^+$ T cells develop in the presence of IL-4 or IL-13, they differentiate into the Th2 subset.[109] In response to extracellular bacteria and fungi, innate immune cells generate large amounts of both TGF-β and IL-6. When naïve T cells receive these signals with additional and sustained IL-21 and IL-23 stimulation, they develop into Th-17 cells

under the regulation of the transcription factor RORγT.[110] The Tfh subset develops in the presence of the cytokines IL-21 and IL-27, which are generated in response to a variety of pathogens.[111] Tregs are exceptionally responsive to IL-2 and can be produced under the influence of environmental factors such as TGF-β and retinoic acid (Figure 10).[112]

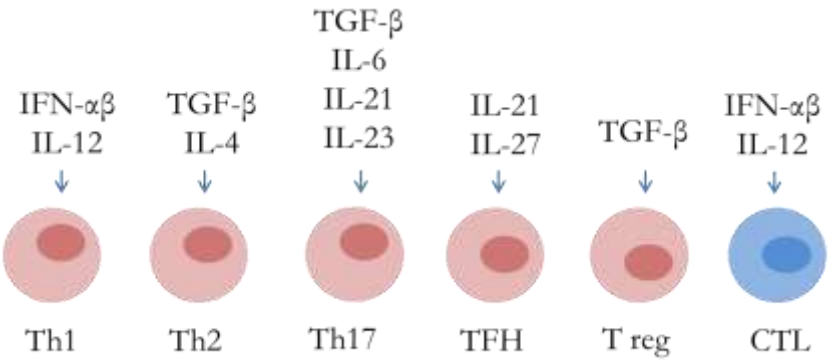


Figure 10. T cell differentiation. DCs recognition of a spectrum of pathogens through various PRR along with TCR engagement results in cytokine release. The microenvironment, thus, plays a central role in the differentiation of the different T cell subsets which are programmed by distinct and unique transcription factors.

1.1.6 The germinal center reaction

The GC are the site of antibody diversification and affinity maturation.[113] GCs form in response to Ag challenge in the center of the B cell follicles of secondary lymphoid organs, interspersed within a network of stromal cells known as follicular dendritic cells (FDCs). The GC reaction is the basis of T-dependent humoral immunity and this is an absolutely essential reaction for any vaccine effect, since the GC will generate the memory B cell population.[114]

FDCs are a non-migratory population found in the B cell follicles in the lymph nodes, spleen and MALT. These cells bind to immune complexes (IC) and in this ways play a critical role in the GC reaction.[115] The FDCs

have high expression of complement receptors CR1 and CR2 (CD35 and CD21, respectively) and Fc-receptor FcγRIIb (CD32). Unlike other DCs and macrophages, FDCs lack MHC II molecules and express few PRR, so they have little ability to capture non-opsonized Ags.[116] The FDCs provide Ags for an extended period of time to enable activated B cell to undergo clonal expansion and differentiation into memory B cells and long lived plasma cells.[115] It has been found that depletion of FDCs dramatically impairs CSR, SHM and memory development in mice.[117,118,119] Moreover, FDCs express receptor ligands and soluble proteins to interact with the activated B cells and provide them with proliferation and differentiation signals.[115,120] For example, CXCL13 has been found critical for the recruitment of activated B cells to the GC.

A second population of cells in light zones of GCs is the Tfh cells. These $CD4^+$ T cells express CXCR5 and access the B-cell follicles in a CXCL13-restricted manner. Although the Tfh represent only a minor population in the GCs, 5% to 20% of all GC cells, they are essential for the GC reaction and for B cell differentiation.[121,122]

Naive B cells continuously recirculate through secondary lymphoid organs and upon Ag-encounter at the B-T cell boundary they become activated and starts dividing in the presence of CD40L-expressing $CD4^+$ T cells. The interaction of CD40, constitutively expressed by the B cells, with its ligand CD40L (CD154), expressed by activated $CD4^+$ T cells, is crucial for the GC reaction. Activated Tfh cells also secrete cytokines that drive B cell proliferation and differentiation and play a central role in triggering the molecular events that lead to SHM and immunoglobulin CSR, and the production of down-stream isotypes, IgG-, IgE- or IgA-expressing B cells.

After initial activation, B cells migrate from the T-B boundary to extra follicular areas, where they are induced to rapidly expand and differentiate

into plasma blasts and plasma cells which provide the most immediate source of Ag-specific antibodies.[123] Ag-engaged B cells remain localized in B-cell follicles and acquire high rates of mutations (in the order of 10^{-3} to 10^{-4} per base pair per generation) in their immunoglobulin variable region (IgV) genes through the process of SHM.[124] GC B-cell clones expressing variants with increased binding to the epitope are selectively expanded, whereas changes that result in impaired Ag binding induce apoptosis of GC B cells.[125] B-cell clones positively selected within GCs differentiate into memory B cells or long-lived plasma cells, which reside in the bone marrow and LP.[126] The memory B cells have changed their gene transcriptional profile to allow for a much more rapid and vigorous response than the naïve B cells in the primary immune response.

1.1.7 Mucosal vaccines

The choice to develop a mucosal vaccine was based on the fact that, although injectable vaccines dominate the market, mucosal immunization is superior for stimulating sIgA antibodies, but it also stimulates significant serum IgG and, most importantly, lung resident cell-mediated immunity. Furthermore, mucosal vaccines appear advantageous compared to injectable vaccines from a manufacturing and a regulatory point of view.[127,128] In general, it can be stated that vaccines that are delivered at the mucosal sites do not require extensive purification from bacterial by-products, whereas production of injected vaccines is rigorous to avoid, for examples, unacceptable endotoxin levels. In addition, mucosal vaccines are practical for mass vaccination and do not involve the risk of spreading blood-borne infections, that can occur with contaminated injection needles.[129,130] The ease of administration, better compliance and the possibility that they can be delivered by personnel without medical training are also viewed as benefits of mucosal vaccines. In particular, mucosal vaccines could be superior for preventing pandemic infections, such as influenza virus infections. Taken together,

mucosal vaccines are highly warranted. However, only very few mucosal vaccines have been approved for human use. In fact, the oral vaccines against cholera, typhoid, polio and rotavirus are the only approved oral mucosal vaccines.[131] Commercial live attenuated flu vaccines given intranasally, such as FluMist, are the only approved intranasal vaccines today.[132-133] Even though there are several advantages associated with mucosal vaccines, the delivery of Ag by mucosal route is a challenge, and it is associated with various problems such as poor immunogenicity, inefficient uptake and presentation by M cells, enzymatic degradation and a risk of developing tolerance, rather than protective immunity. Therefore, the selection of the route of administration, an adjuvants and a potent delivery system are very crucial to achieve more effective mucosal vaccines.[134]

Route of administration

When deciding on which route to deliver a vaccine, it has to be taken into account the anatomical, functional, and immunological features of the different tissues.[135] The anatomical structure and spatial organization of the tissues, the presence of mucus, peristalsis in the intestine and physical discharge in the respiratory tract must all be considered and evaluated. Safety issues have also to be considered.

Oral vaccines represent the biggest challenge for mucosal vaccine development. This is because of the harsh gut environment, which degrades most antigenic epitopes that are delivered in soluble form. Another factor that impairs oral vaccination is the fact that immune suppression or tolerance can develop. Intranasal vaccination stimulates immune responses in the NALT and is effective at inducing systemic and mucosal immunity in the respiratory and genital tracts. In general, intranasal vaccination is an attractive approach, as much lower Ag and adjuvant doses are required compared with oral vaccination. The

sublingual route for vaccine administration is also gaining interest because vaccine delivered via this route stimulates strong mucosal IgA and systemic IgG antibody responses as well as cytotoxic $CD8^+$ T cell responses.[136]

An important notion in this context is that the response to mucosal vaccination is mostly compartmentalized. In this way, intranasal immunization provides poor immunity to the gastrointestinal tract, while oral immunization does not protect against lung infections and vice versa. Indeed, the presence and localization of particular DCs sub-sets impact on the outcome of mucosal vaccination.[136]

Adjuvants

"Adjuvare" in Latin means "to help". Thus, adjuvants are molecules used to induce more potent and long-lasting protective immune responses. The addition of an adjuvant to vaccines enhances the immunogenicity of the vaccine. In some cases, this also means reducing the required doses of Ag for a significant immune response.[137] Therefore, an ideal mucosal adjuvant should enhance strong humoral and effector and memory B and T cell responses.[138] The adjuvants used for mucosal vaccination are many, but few have found a commercial application. These adjuvants are essentially molecules like bacterial toxins, cytokines, TLRs, which are capable of augmenting immune responses.

The bacterial enterotoxins cholera toxin (CT) and the closely related *Escherichia coli* heat labile toxin (LT) have been given the status of golden standards for a mucosal vaccine adjuvant.[139] These toxins are AB_5 complexes in which the A subunit is composed of the A1 portion (with ADP-ribosyl transferase activity) and the A2 chain that merge with the B subunit pentamer. The B subunits are responsible for binding to gangliosides that are present on the cell membrane of all nucleated cells.

Whereas the B subunit of CT binds to GM1 ganglioside, the B subunit of LT can also bind to other gangliosides, such as GM2.[140,141] Although CT and LT act as the most potent mucosal adjuvants we know of today, their use in humans is precluded because of their high toxicity. To overcome the toxicity various approaches has been taken. Several non-toxic or attenuated CT and LT molecules have been generated and tested for their ability to enhance immune responses. The CTA1-DD adjuvant is such a non-toxic and safe adjuvant derived from CT and it was used in this research project. The CTA1 gene, encoding for the ADP-ribosylating enzyme, was fused to a dimer from the Ig-binding *Staphylococcus aureus* protein A. Because CTA1-DD fails to bind to the GM1 ganglioside receptors, it is also non-toxic and safe.[142-144]

Another family of adjuvant molecules is those that bind to the TLRs. These adjuvants are potent enhancers of the immune response through activation of the innate immune response. Monophosphoryl lipid A (MPL) for example is derived from LPS of Gram-negative bacteria, such as *Salmonella Minnesota*. LPS stimulates innate immunity via TLR-4 on APCs.[145] MPL is usually used in complex formulations that include liposomes and emulsions to induce enhanced T-cell responses.[146] Synthetic oligodeoxynucleotides are another TLR-based mucosal adjuvant, hosting un-methylated cytosine-phosphate-guanine (CpG) dinucleotides.[147] Mucosally delivered CpG can stimulate pro-inflammatory and Th1-inducing cytokines by binding to TLR-9.[148] Another example of TLR-based adjuvant is bacterial flagellin which activates via TLR-5.[149]

Also purified cytokines can be used as potential adjuvants. These are, among others, interferons and granulocyte-macrophage colony-stimulating factor (GM-CSF). Because these cytokines are short-lived and can induce dose-related toxicity, they are difficult to apply to clinical vaccination.[150] For example, intranasally administered IFN-α/β can

increase resistance against influenza virus, but in human trials they failed to improve the performance of a trivalent flu vaccine.[151] Another example is intranasal administration of IL-12, which can enhance activation of Th1-associated functions and contribute to IFN-γ dependent Ig-production, but this is also not clinically feasible on a broader scale.[152,153]

Delivery systems

Delivery systems such as microparticles, liposomes and other particulates enhance the immune response based on their particular characteristics. An ideal mucosal delivery system should protect from enzymatic digestion, induce efficient uptake and presentation of Ags by DCs, be non-carcinogenic and non-pyrogenic and stable in wide range of pH intervals. Various delivery systems used for mucosal immunization have been developed including inert systems such as microparticles, liposomes, ISCOMs, and different live attenuated bacterial or viral vector systems.[134]

Polymer-based delivery systems have been widely used as carriers for protecting Ags from the harsh proteolytic conditions of the gastrointestinal tract and facilitate their subsequent release in the intestine. In vaccines these polymeric materials allow the release of Ag in a controlled manner thereby reducing the frequency of vaccinations and promote interaction with mucosal epithelium.[154] Liposomes are made up of phospholipid and have been widely used as carriers of Ags. The immunogenicity of liposomes depends on lipid composition, size, surface characteristics, and on the chirality of lipids. Liposomes can protect the loaded Ag from enzymatic degradation, and can deliver the Ag to a specific cell type, provided that the surface can be equipped with specific cell targeting molecules.[155,156]

1.1.8 Vaccination in adults vs neonatal vaccination

Before going into detail about what is vaccination, it is useful to take a step back and focus on immunization in general, i.e. the process by which an individual's immune system becomes fortified against an agent (known as the immunogen). Improving the immune status of an individual could involve both passive and active immunization. Whereas **passive immunization** involves the transfer of preformed antibodies to a naive host, the **active immunization** (vaccination) stimulates an endogenous immune response to injected or mucosally administered Ags inducing the generation of long-term memory and long-lived plasma cells.[157] Passive immunization occurs, for example, when antibodies from the mother passes through the placenta to the fetus.[158] An active immunization generates an immune response that could last much longer than passive immunization.[159,160]

Vaccination of neonates

Talking about vaccination, it is always very important to keep in mind which is the target population of a specific vaccine. The immune system of neonates and infants is significantly different from that of the adult. This contributes to the increased risk of morbidity and mortality among neonates, who can acquire many types of infections. Neonates are exposed only to few Ags in utero, and, therefore, they lack an adaptive immune response that can convey protection. Hence, neonates are completely reliant on passive immunity and maternal antibodies as their first line of defense. As the neonate undergoes microbial colonization of the mucosal membranes and the intestinal tract, in particular, the systemic and local immune systems mature rapidly.[161] The innate immune system of the neonate is functionally impaired compared to that of the adult. For example, blood-derived monocytes from human infants have reduced production of cytokines such as IFN-α, IFN-γ and IL-12 upon TLR

stimulation, but produce increased levels of IL-10 and the Th17-inducing cytokines, such as IL-6 and IL-23. Antiviral cytokines, such as type I IFNs, are protective in young children.[162,163,164,165,166] Neonates also have altered adaptive responses compared to adults. Antibody responses are reduced and of shorter duration. The antibodies often have a poor affinity for the Ag and the IgG isotype skewed away from IFN-γ influenced IgG-subclasses towards Th2-skewing. [167,168,169] In fact, not only the frequency of T cells is decreased in neonates, but also the quality of the response is altered compared to that observed in adults. The majority of peripheral T cells in neonates are recently emigrated from the thymus and they are defective in Th1 responses. These differences are thought to explain why neonates suffer from poor protection against infections. In addition an altered inflammatory response has also been observed in neonates. Yet, another possibility is that neonatal T cells may differ in the diversity of their TCR. During T cell development, diversity is accrued in the TCR repertoire by VDJ recombination and non-coding nucleotide insertions and deletions in the complementary determining region (CDR) 3 of the TCR. These CDR3 insertions and deletions are mediated by the enzyme terminal deoxynucleotidyl transferase (TdT), which is responsible for up to 90% of the TCR diversity.[170] In humans this enzyme is expressed in the thymus during the third trimester, but in mice it is turned on between days 4 and 5 of age. As mice age, the CDR3 length of their TCR increases, but shorter CDR3 regions have been associated with increased cross-reactivity and lower affinities to peptides.[171,172] Because of these shortcomings in the neonatal immune system, only three vaccines have been administered to newborns (first 24 hours of life): the hepatitis B virus (HBV) vaccine, the Bacillus Calmette–Guérin (BCG) vaccine and the oral poliovirus vaccine.[173,174]

1.2 Influenza virus and the importance of a universal vaccine

1.2.1 Overview of Influenza virus biology

Influenza is one of the major viral disease affecting humans and it is responsible for three to five million cases of severe illness and about 250.000 to 500.000 deaths each year worldwide. Typically, the virus infects about 10% of the world population during seasonal epidemics, i.e. more than 500 million people (World Health organization, WHO). Moreover, due to the risk of fast and global spread of the infection, as we saw with the 2009 swine-flu, a vaccine against pandemic influenza infection is much warranted and mass vaccinations are required to limit the severity and spread of infection.

Influenza virus is a member of the *Orthomyxoviridae* family of RNA viruses, and it is an enveloped virus. There are three types of influenza viruses: A, B and C. The Influenza virus A stains are responsible for pandemic infections and they can infect humans, as well as many other mammals and birds. They are further classified into subtypes based on the antigenic structure of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA).[175] To date, scientists have identified 16 HA and 9 NA subtypes.[176] Influenza viruses are pleomorphic virus, as they can vary in size and shape. Influenza virus A virions have a spherical morphology with a diameter of about 80-120nm. They are characterized by a lipid envelope with glycoproteic spikes. Below the lipid envelope there is a layer of matrix protein consisting of the M1 protein that provides support for the viral envelope. This additional layer encloses the nucleocapsid which consists of ribonucleoprotein (RNP) and hosts the genome. The viral genome is composed of eight segments of single-stranded negative sense RNA that have to be copied into single-stranded

positive sense mRNA to ensure protein synthesis. The presence of a segmented genome gives the virus the opportunity to undergo considerable variations in the different structural proteins and, therefore, in the antigenic composition (Table 1).[177]

The virus initially binds via the HA glycoprotein to its receptor 5-N-acetyl neuraminic acid (sialic acid) on the surface of the host cells.[178] The virus is then rapidly internalized into clathrin-coated pits. This process is dependent on dynamin, a cellular GTPase.[179] The incorporated virus traffics through the endocytic pathway and ultimately reaches a low-pH compartment of approximately 5.5.[180] At this pH, the viral fusion machinery is triggered. HA undergoes a conformational change, forming a 'coiled-coil' of α-helices and exposing the previously buried hydrophobic fusion peptide, which is then inserted into the endosomal membrane. The presence of the M2 ion channel in the envelope of the virus allows for the components inside the virus to become exposed to the low pH of the endosome, which leads to the disruption of M1–vRNP interactions and the uncoating of the virus.[181] This initiates the fusion event and the release of the interior components of the virus, i.e. M1 and RNPs, into the cytoplasm. The import of vRNPs occurs through nuclear pores and is mediated by nuclear localization signals on NP.[182]

Seg.	Prot.	Bp	MW (Da)	Function
1	PB2	2425	86.000	PB1, PB2 and PA constitute the so-called transcriptasic complex which is formed by the subunit of the viral RNA dependent-RNA polymerase.
2	PB1	2341	83.000	
3	PA	2233	85.000	
4	HA	1778	80.000	HA is the receptor that binds the virion to sialic acid residues present in glycoproteins and glycolipids in the membrane of target cells.
5	NP	1565	56.000	NP has high affinity for RNA, maintains the morphology of the nucleocapsid and allows the translocation of the viral genome from the cytoplasm to the nucleus of the target cell.
6	NA	1413	50.000	NA is able to cut the sialic acid residues present on the membrane of the newly infected cell.
7	M1	1027	27.000	M1 interacts with the nucleocapsid during the stages of maturation.
	M2		11.000	M2 is able to modulate the pH inside the virion by acting as a proton channel, thus participating in the uncoating process.
8	NS1	890	27.000	NS regulates the transport of mRNA, the splicing process and the translation process.
	NS2		13.000	

Table 1. Outline of the influenza genome segments and proteins function.

Influenza virus represents one of the few RNA viruses that undergo replication and transcription in the nucleus of their host cells.[183] In the nucleus, the vRNPs serve as templates for the production of two forms of positive-sense RNA, namely viral messenger RNA (mRNA) and complementary RNA (cRNA). The synthesis of mRNA is catalyzed by the viral RNA-dependent RNA polymerase (composed of the three

subunits PA, PB1 and PB2). Viral mRNAs are capped (i.e. contain a methylated 5' guanosine residue), polyadenylated (i.e. contain a sequence of polyadenylic acid at their 3' end), and exported from the nucleus to undergo translation. The nuclear export of viral mRNA utilizes the host cell machinery and is controlled by the viral non-structural protein NS1. Many viral proteins (NP, M1, NS2 and the polymerases) are then imported into the nucleus for the final stages of replication and for vRNP assembly. The viral cRNA is neither capped nor polyadenylated and remains in the nucleus, where it serves as a template for the production of negative-sense genomic RNA (vRNA). The newly formed vRNPs are exported from the nucleus. This process appears to be a reversal of the nuclear import process because it occurs through nuclear pores. M1 is a major regulator of nuclear transport. The translocation event might depend on nuclear export signals on the NS2 protein, or even on NP itself.[184] For virus assembly at the plasma membrane, it is essential that all of the viral components (i.e. HA, NA, M2, M1 and the vRNPs) are present at the correct location in the cell and are correctly processed. [185] M1 molecules bind to vRNPs, to the plasma membrane and also to other M1 molecules to form a shell beneath the virus envelope.[186]

The formation of new viruses appears to rely on the presence of the cytoplasmic tails of both HA and NA. These glycoproteins, along with M1, M2 and host-cell factors (the actin cytoskeleton and the polarized nature of the cell), control the morphology of the new particles.[187] In polarized epithelia, budding of virus particles occurs exclusively from the apical surface. The final release of viruses from the cell surface relies on the action of the viral NA. NA (sialidase) acts as a receptor-destroying enzyme, by removing sialic acid from the surface of host cells. Without this step, the newly formed virus particles would immediately re-bind to their receptor and would not be released into the extracellular space.[188]

Two closely related anti-viral drugs that block influenza A virus infections, i.e. amantadine and rimantadine, target the pH-dependent uncoating event and disrupts the function of the M2 channel, preventing acidification and inhibiting M2-dependent virus uncoating.[189] Furthermore, two analogues of sialic acid, namely zanamivir and oseltamivir, are effective against both influenza A and B viruses because they function as inhibitors of NA preventing virus release and spreading from cell to cell.

1.2.2 Influenza virus epidemiology and tropism

Influenza epidemiology relies on the ability of the virus to give rise to variable and antigenically new strains. Genome changes can occur by two distinct mechanisms: the antigenic shift and antigenic drift. The antigenic drift consists in a modification of the surface proteins (that are capable of stimulating an immune response) amino acids sequences. It is due to the introduction of a high number of errors during the genome replication by RNA dependent-RNA polymerase that lacks the 5'-3' exonuclease (proofreading) activity; moreover, the selective pressure elicited by the host immune system induces the survival of resistant virus isolates. It affects both influenza viruses type A and B, but in A viruses is more heavy and frequent; it is responsible of seasonal epidemics. New variants become sufficiently unrecognizable to antibodies of the majority of the population to make a large number of individuals susceptible to the "new" mutated strain.[190] The antigenic shift only affects influenza virus type A and is fortunately less frequent. It consists in the appearance within the human population of a new viral strain with surface proteins belonging to a different subtype than those commonly circulating in humans. The antigenic shifts are due to the reassortment between human and animal viruses (avian or swine) or to the direct transmission of non-human viruses to humans.[191] However, it is important to emphasize that the appearance of a virus strain with radically new surface proteins is not

sufficient. Efficient spreading from human to human is also necessary. *In vivo*, the principal cell types targeted by influenza viruses are the epithelial cells of the respiratory mucosa. When the virus is inhaled, it encounters the apical face of columnar epithelial cells. Following replication, the virus is also released from the apical face of the cell into the airways. The lack of basolateral release generally precludes the systemic spread of influenza viruses in their host. Released viruses can spread from cell to cell, be exhaled and infect a new host. They can also be recognized by cells of the immune system, including alveolar macrophages, which engulf and destroy the virus, and circulating DCs, which migrate out of the lung tissue and present viral Ags to T cells. *In vitro*, viruses are typically studied either in embryonated chicken eggs or in Madin-Darby canine kidney (MDCK) cells, both of which support the multi-cycle growth of influenza viruses. Many other cell types can be infected by the virus, but undergo only a single cycle of infection without spreading virus from cell to cell.[192]

1.2.3 Immune responses to influenza virus infection

The innate immune responses to influenza virus infection have been well characterized. The virus infection is sensed by PRRs that react to viral RNA. Signaling via these receptors triggers the production of pro-inflammatory cytokines and type I interferons (IFN-α and IFN-β).[193] Type I interferons induce interferon stimulated genes (ISGs) via the JAK/STAT signaling pathway.[194] They have strong antiviral activity because they inhibit protein synthesis in the infected host cells and limit virus replication. Type I interferons also stimulate DCs resulting in enhanced Ag presentation and priming of $CD4^+$ and $CD8^+$ T cells, thereby driving adaptive immune responses.[195] Moreover, alveolar macrophages become activated and phagocytize apoptotic influenza virus-infected cells, which also limits viral spread.[196] These cells produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNFα),

which are partially responsible for the immunopathology seen with influenza virus infections.[197] Another important innate response is that of NK cells that react to antibody-bound influenza virus infected cells and lyse these cells through antibody-dependent cellular cytotoxicity (ADCC).[198]

The adaptive immune response to influenza virus is initiated by DCs below the airway epithelium. These cells take up whole virus or virus Ags and migrate to the draining lymph nodes. They also take up apoptotic bodies from infected cells and degrade viral proteins to peptides that can be presented to T cells in the context of MHC I or class II molecules.[199] In this way, both $CD4^+$ T cells as well as $CD8^+$ T cells with CTL activity will be induced. It has even been reported that DCs could exert cytolytic activity themselves against infected host cells.[200]

Influenza virus infection stimulates specific antibody responses of all isotypes. They are directed mainly against the two surface glycoproteins HA and NA. The HA-specific antibodies predominantly bind to its trimeric globular head and inhibit virus attachment and entry into the host cells. Indeed, the presence of antibodies against these proteins correlates well with protection.[201] Secretory IgA antibodies are produced locally, and have been documented to afford protection against infection by neutralizing influenza virus in infected epithelial cells.[202,203] Serum IgM antibodies are produced rapidly in response to infection and are a hallmark of a primary infection.[203,204] Serum IgG afford long-lived protection and are involved in phagocytosis of infected cells as well as ADCC mediated protection.[205] In contrast to the relatively variable HA globular head, the HA stem region is highly conserved mainly because it is physically masked for the immune system. Importantly, some anti-HA stem-specific antibodies have been found to bind to HA molecules from many different subtypes and have broad neutralizing capacity, making them highly interesting from a universal vaccine persective.[206]

Furthermore, antibodies against NA also have protective potential and facilitate ADCC as well as enhance viral clearance.[207] Another interesting protein for a universal vaccine is M2, which is highly conserved among mammalian as well as avian influenza virus strains. Whereas natural infection stimulates only low serum titers against M2, active immunization with the M2e-ectodomain is highly effective.[208] Also NP-specific antibodies contribute to protection.[209,210]

Cell-mediated protection against influenza infection is well documented and prospects are that broadly protective vaccines need to stimulate both $CD4^+$ and $CD8^+$ T cell immunity. The reactivity of memory T cells is directed against many different Ags and epitopes, including the NP, M1 and PB1 proteins, but also other proteins may be involved.[211,212] These proteins are highly conserved and therefore T cell responses display a high degree of cross-reactivity, even between different subtypes of influenza A virus. This is why a universal broadly protective influenza vaccine will carry several of these epitope specificities. Whereas we mostly associate CTL activity with $CD8^+$ T cells, recent studies have identified that also influenza-specific $CD4^+$ T cells can exert cytolytic functions.[213] The lytic activity of CTLs is dependent on perforin and several granzymes, where granzyme B is important. Whereas perforin permeabilizes the cell membrane of the infected cell, granzymes enter the cell and induce apoptosis.[214] Another mechanism whereby CTLs can induce apoptosis of target cells is mediated by Fas/FasL interactions. Most of the influenza-specific $CD4^+$ effector T cells of the Th1 and Th17 subsets are known to contribute to protection through cytokine production. The Th1 cells produce IFN-γ, which has been found critical for protection against influenza.[215] In addition, IL-17 and other cytokines from Th17 cells have been found effective in protection against influenza virus infection, although the precise mechanism is presently poorly understood. In an inflammatory environment, Th17 cells improve T

helper responses by producing IL-6, which inhibits Treg-mediated suppression, which secures stronger immune effector T cell responses. [216-217]

Following resolution of primary infection, a subset of responding virus-specific $CD4^+$ T cells are retained as long-lived memory T cells which can persist and convey protective immunity upon Ag re-encounter. Like effector T cells, all memory $CD4^+$ T cells retain high-level expression of CD44. However, memory T cells are heterogeneous in their expression of CD62L and CCR7 which lead to the delineation of the two memory subsets: $CD62L^+CCR7^+$ TCM and $CD62L^-CCR7^-$ TEM cells.[218] Following influenza infection, virus-specific TEM are recirculating and predominant in the circulation and peripheral tissues, including the lungs, while TCM are predominant in lymph nodes. Both populations are present in the spleen.[219] In addition to TEM and TCM subsets, a distinct population of non-circulating $CD4^+$ and $CD8^+$ memory T cells has been identified. TRM cells reside in the tissues and persist long-term after infection.[200,220] The TRM provide early protection and CTL activity in the tissues within days from an influenza virus infection.[221] Phenotypically, $CD4^+$ TRM can be distinguished from circulating TEM and TCM by upregulated CD69 and CD11a expression.[222] $CD8^+$ TRM express CD69 as well as the integrin CD103, which is not significantly upregulated by $CD4^+$ TRM.[223] In mouse models of influenza infection, a population of virus-specific $CD4^+$ TRM persists in the lungs for long periods following infection.[224]

1.2.4 Seasonal vaccination and the challenges of a universal influenza vaccine

Vaccination is an efficient method to prevent influenza virus infection. Seasonal influenza vaccines are safe and reduce the impact of the annual influenza epidemics. These vaccines have until recently been produced in

embryonated eggs and, after enrichment of the virus and inactivation by formalin or propriolactone and ultra-centrifugation, are split using a detergent.[225] The HA content is then quantified by the single radial immuno-diffusion assay.[226] These inactivated influenza vaccines are given as injectable vaccines, but there also exists a live-attenuated influenza vaccine (LAIV) that is given as an intranasal spray vaccine (Table 2).

Because of a high probability for gene reassortments and the antigenic diversity among primarily swine and avian influenza virus reservoirs, we face a risk of new emerging strains that can cause pandemic infection. This means exceptional challenges for influenza vaccine design and development to get to a broadly protective universal vaccine against influenza. To prepare for future pandemic outbreaks of influenza A viruses, new influenza vaccines are under development. [227] The Achilles' heel of today's seasonal influenza vaccines is that they provide limited protection against pandemic influenza strains.[228] The WHO coordinates an international surveillance system to monitor circulating influenza viruses isolated from both humans and animals, (especially birds and pigs) and detect newly evolved antigenic variants.[229] Currently, two subtypes of influenza A virus (H3N2 and H1N1) and one strain of influenza B virus are responsible for outbreaks of human disease and are, therefore, included in the seasonal influenza vaccines.

	Inactivated vaccine	Live attenuated vaccine
Effect	Generation of antibodies (IgG) against HA.	Generation of antibodies (IgG and IgA) against a number of specific gene segments such as M2, PB2, and NS1.[230]
Formulation	- Whole virion. - Split virion derived by disrupting whole virus particles with disinfectants. - Subunit form prepared by enriching for HA and NA following disruption of viral particles.	HA and NA of the target strain is introduced into the backbone, cold-adapted virus.[231]
Route of administration	Parental injection.	Intranasal inoculation of replication competent virus.
Advantages	- Efficacy of 70% in the age group between 14–60 years.[232] - Safety.	- Antigenic phenotype of the target strain but the attenuated phenotype of the master strain. - Induction of both a local immune neutralizing antibody and a cell mediated response.
Disadvantages	- Efficacy is largely dependent on the degree of matching between vaccines and circulating strain. - Efficacy is reduced with both infants and the elderly. [233] - Immunity generated is not particularly broad. - At least two doses of vaccine must be provided to generate protective immune responses in naïve individuals. [234] - Length of time between selection of vaccine strains and the availability of the first doses of formulated vaccines. [235]	- Possible reversion to virulence. - Requirement of two doses to elicit optimal immune responses. - Possibility that not all HA and NA combinations will form viable viruses on the attenuated backbone. - The vaccine virus must be able to infect the human upper respiratory tract .[236] - Potential safety risks of administering a live virus.

Table 2. Features of conventional influenza vaccination approaches.

The major challenge with the development of a broadly protective universal influenza vaccine is to identify conserved protein regions or epitopes to include in the vaccine. One of the most studied vaccine epitopes for a universal vaccine is the extracellular portion of the M2 protein (M2e).[237] In addition, conserved domains in the stalk region of HA have been explored, as well as a number of other proteins, such as M1, NP and PB1.[238] Also, lowering the time needed for manufacturing of the vaccine is investigated and production of influenza vaccines in cell cultures has provided promising results. However, most critical is to achieve sufficient breadth of the specific immune response. Exploring plasmid-based reverse genetics systems has had important impact on the field.[239] This allows for the generation of viruses of well-defined genetic composition within a shorter time frame and provides advantages in the development of seed strains. Another strategy is to use DNA vaccines expressing various combinations of the viral HA or NA as well as other viral genes.[240] The production of DNA vaccines is safe, economic and rapid. However, DNA vaccines are typically hampered by low immunogenicity, particularly in larger animals and humans. Better methods of DNA delivery are currently under development.[241,242]

1.3 Protein-based vaccines and nanoparticle formulation

1.3.1 CTA1-DD, a fusions protein-based adjuvant vector

Whereas a live attenuated influenza vaccine provides sufficient immunogenicity in itself, inactivated or subcomponent vaccines require potent adjuvants. This does not only apply to injectable vaccines, but it is the main reason for the low number of mucosal vaccines on the

market.[136] Many different adjuvant formulations have been or are being tested for improvement of influenza vaccines. We have developed a subcomponent vaccine against influenza virus, CTA1-3M2e-DD, consisting of an adjuvant moiety (CTA1), the selected vaccine epitope (M2e) and a cell-targeting unit (DD). The CTA1-DD adjuvant is effective at mucosal as well as systemic sites. It is potent and non-toxic and composed of the ADP-ribosylating moiety of cholera toxin (CT) and a dimer of *Staphylococcus aureus* protein A (DD).[243,244] CTA1-DD has been shown to be non-toxic and safe in experimental animal models, including non-human primates. It acts broadly and enhances a wide range of antibody, $CD4^+$ T cell and cytotoxic $CD8^+$ T cell responses following intranasal immunizations.[142, 245] It exploits the full immunomodulating ability of CTA1 in a fusion protein that is effective at targeting DCs via the DD-moiety (Figure 11).

The bacterial enterotoxins CT and *E. coli* heat-labile toxin (LT) share a high degree of homology and are exceptionally potent mucosal and systemic adjuvants. These enterotoxins bind via their B subunits to GM1-ganglioside, which is present on the cell membrane of all nucleated cells. This is why they are rather promiscuous and carry unwanted side effects given orally or intranasally, resulting in diarrhea and blocking nerve functions, i.e. in Bell´s palsy, a transient facial nerve paralysis, respectivly.[246] For this reason, mutant enterotoxins or derivatives of these toxins have been developed and found significantly safer adjuvants. The CTA1-DD adjuvant is an example of an effective derivative of these enterotoxin adjuvants.[243,244,142, 245]

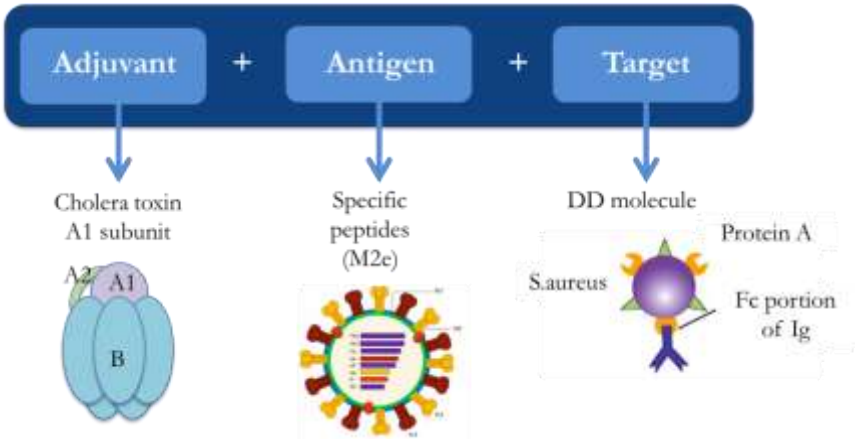


Figure 11. CTA1-X-DD fusion protein.

In this project we have investigated how to optimize the formulation of CTA1-3M2e-DD and other fusion proteins into nanoparticles using the liposome or polysaccharide technologies.

1.3.2 Nanoparticles and the immune response

Subcomponent vaccines have been found more effective if formulated in particles, mimicking their representation in the native microorganism. The aim of the nanoparticle formulation is, thus, to enhance and promote an effective Ag uptake, especially across the mucosal barrier.[247,248] Various materials can be used to produce nanoparticles, including polymers, lipids, proteins or metals, such as gold. Important factors to consider for the successful manufacturing of nanoparticles are the Ag load, size, charge, cell target and the desired outcome of the immune response.[249,250,251,252,253] Although nanoparticles with different characteristics have been extensively used for vaccine delivery, their mechanism of action and how to optimize their design remain unclear. It is well documented that immune responses to nanoparticles are differently modulated depending on the different formulations used. However, it is difficult to dissect the contribution of individual properties

of a formulation, such as size or charge, because changing one element usually influences several features and functions. Surface charge can be modified by altering the lipid formulation in liposomes, but this also affects other properties, such as particle rigidity and stability. Hence, it may be difficult to directly assess the influence of changing one parameter when assessing physicochemical properties and immunogenicity of different nanoparticles.[254]

Charge matters

One of the most critical parameters is the charge of the nanoparticle. This is assessed by the zeta potential, which is a measure of the electrostatic potential at the limit of a diffuse layer of differently charged ions spatially distributed at the surface of the particle. The magnitude of the zeta potential, thus, depends on the concentration of ions within the double layer, but also other factors, such as the ionic strength and pH of the dispersion medium. Because the cell surface, as well as the mucus coating of the mucosal membrane, are negatively charged, it is frequently hypothesized that positively charged nanoparticles will exhibit stronger interactions with the cell membrane, as well as provide increased mucoadhesion. The latter leads to reduced clearance rate, i.e. slower removal from the mucosal membranes, which is thought to enhance immunogenicity. This is because increased interactions with the cell membrane and a prolonged exposure time to the Ag lead to increased Ag uptake. In general, positively, cationic, charged nanoparticles have been shown to be better retained and more immunogenic at mucosal membranes than negatively charged or neutral nanoparticles.[255,256] Furthermore, cationic nanoparticles were found to effectively deliver Ag to both mucus and APCs.[257,258] On the other hand, negatively charged nanoparticles have been shown to exert an immunosuppressive effect on alveolar macrophages and thereby promoting an enhanced immune response.[259-261,261-262,263] Thus, several mechanisms are modulated simply by

changing the charge of the nanoparticle, which also will affect the fate when nanoparticles are given by different routes that often differs in the charge of the microenvironment.

Size matters

Nanoparticles with varying sizes have been found to have different effects following mucosal immunization.[264] There is an extensive literature analyzing the effect of particle size on the immune response. The most consistent result shows an advantage of nanoparticles (smaller than 200 nm), over microparticles (>1μm), at priming cytotoxic $CD8^+$ T cells (Figure 12).[265] A common perception is that nanoparticles resembling the dimensions of viruses are processed like viruses and induce a strong $CD8^+$ T-cell response, whereas microparticles, being closer to the size of bacteria, induce stronger humoral immunity.[266] The explanation might be that micron-sized particles can be taken up through receptor-mediated endocytosis and phagocytosis, but their size may restrict macropinocytosis. DCs have an exceptional capacity for macropinocytosis and therefore may favor the uptake of nanoparticles over microparticles.[84,267] On the other hand, macrophages effectively take up microparticles. As a consequence, DCs are more prone to stimulate $CD8^+$ T cells by cross-presentation, while macrophages are favoring MHC II over MHC I presentation.[268,269,270]

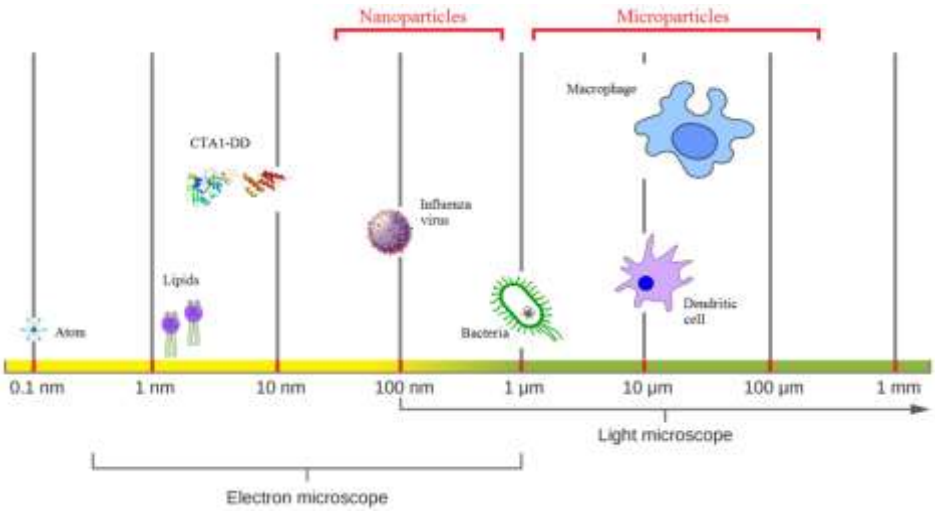


Figure 12. Examples of particles, vaccine components, pathogens, cells and their approximate size range.

The molecular composition matters

The molecular composition is critically influencing not only biological features, but also the stability of the nanoparticle. A more stable formulation usually leads to delivery of larger Ag loads which can contribute to a depot effect. Liposomes are a good example of this as various lipid combinations can impact on nanoparticle stability. For example, nanoparticles with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), having a higher transition temperature, were more stable *in vitro* and likely were better protected from degradation in the gastrointestinal tract.[271] Moreover, stable nanoparticles containing 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine (DPPS) induced stronger IgA responses compared to formulations without DPPS.[272] However, it is important to acknowledge that changing the lipid composition also alters the charge, which in itself may impact greatly on the performance of the nanoparticle.[273]

Antigen compartmentalization matters

Ags can be carried in many different compartments of the nanoparticle. This impacts greatly on the immunogenicity of the Ag. For example, Ag can be limited to the aqueous core of the nanoparticle, or inserted into the membrane leaflet, or bound to the surface by covalent bonds or intermolecular forces. Thus, the nanoparticle formulation may be tailored for specific vaccine needs. For an oral vaccine encapsulating the Ag inside the nanoparticle has proven an effective strategy to prevent Ag-degradation. On the other hand, by hiding the Ag inside the nanoparticle the immunogenicity may be compromised as specific antibodies could be difficult to rise. Moreover, the i.n. route is less exposed to Ag degradation compared to the oral route, which could apply also to the adjuvant incorporated into the nanoparticle. It is generally thought that the adjuvant exerts its most important function in DC-priming of T cells and should be encapsulated. However, at variance with this notion, cholera toxin B-subunit (CTB) adjuvant bound to the surface of the nanoparticle was more effective than when encapsulated into the nanoparticle.[274]

Surface modifications matter

Considerable attention has been given to study how nanoparticles are retained by and/or taken up across the mucosal membranes. Many different strategies have been tested to enhance the ability to penetrate mucus and to be retained at the mucosal membranes. To increase membrane adhesion of the nanoparticle chitosan has been explored. It was clearly found that chitosan stimulated enhanced IgG antibody responses.[275] Chitosan is a positively charged polysaccharide that can form strong electrostatic interactions with cell surfaces and mucus and, therefore, can increase retention time and facilitate interactions between the nanoparticle and the APCs. Nanoparticle interactions with the

intestinal mucosa have been studied *in vivo* and *ex vivo* using many different models.[276-278,276, 279]

The most used way to retain circulating liposomes is to sterically stabilize the liposome surface by lipid-conjugated polyethylene glycol (PEG).[280] PEG is a linear non-immunogenic and hydrophilic polymer and it has been approved by FDA for human use. It is hypothesized that the presence of hydrophilic polymers, such as PEG, on the surface of liposomes attracts a water shell, resulting in reduced adsorption of opsonins and retained presence in the circulation. This, in turn, results in a decrease in both the rate and extent of uptake of liposomes in APCs. On the other hand, at mucosal membranes it was noted that PEGylation resulted in better mucus-penetrating nanoparticles and augmented immune responses.[281] PEG was shown to accelerate the drainage of liposomes into LNs, with a prolonged retention in APC, which improved vaccine efficiency.[282] Significantly higher specific IgA and IgG antibody levels were found with PEGylated than with un-PEGylated nanoparticles.[281] Moreover, PEG reduces protein aggregation owing to repulsion between PEGylated surfaces and increases thermal stability of proteins. However, a reduction in biological potency has also been reported after PEGylation.[283] Currently, several preparations with increased retention time are commercially available, such as Doxil®, doxorubicin-containing PEGylated liposomes.[284]

Cell-targeting matters

Many different cell-targeting strategies with nanoparticles have been attempted. For example, specific antibodies have been found to enhance binding to M cells, thereby targeting the nanoparticle to the FAE.[285] Similarly, lectin Agglutinin I from *Ulex europaeus*-coated nanoparticles were shown to improve M cell-targeting and Ag uptake.[278, 286-287] Also, galactosylation of nanoparticles resulted in higher specific IgA and IgG

antibody levels compared to unmodified nanoparticles.[288] Moreover, nanoparticles coated with the influenza virus protein HA were more immunogenic than uncoated nanoparticles.[289] In addition, mannosylated lipids or anti-CD40 antibody-coated nanoparticles were found to host an enhanced ability to target DCs and, thereby, greatly promoted stronger immune response.[290,291] Furthermore, the identification of Mincle, a receptor for the mycobacterial cord factor trehalose 6,6'-dimycolate (TDM), on innate immune cells led to that TDM analogs were found to be effective stimulants of the production of Granulocyte colony-stimulating factor (G-CSF) in macrophages. Indeed, immunizations in mice with cationic nanoparticles containing the analogues TDM demonstrated superior adjuvant properties. [292]

DEC-205, Clec9A and Clec12A are DCs receptors that are promising target for particle-based vaccination.[293] DEC-205 is a C-type lectin receptor expressed in mice on thymic epithelial cells and DCs. In humans DEC-205 is also found on moDCs, monocytes, B cells and NKT cells.[294,295] The DEC-205 receptor binds dying cells for uptake and cross-presentation of debris-associated Ags.[296] Several reports have demonstrated that ex vivo targeting of mouse DCs, and more specifically $CD8^+$ DCs, with ovalbumin (OVA)-conjugated anti-DEC-205 antibodies induces robust MHC-I cross-presentation to OVA-specific $CD8^+$ T cells. Furthermore, these conjugates elicit high OVA presentation by MHC-II molecules.[297] Clec9A (also known as DNGR-1) and Clec12A are also C-type lectin-like receptors expressed in mice on $CD8^+$ DCs.[298,299] Clec9 recognizes an actin-containing cytoskeletal structure that is exposed on apoptotic and necrotic cells when the cell membrane is ruptured.[300] *In vivo* injection of anti-Clec9A antibodies conjugated to an MHC-II-binding OVA peptide together with an adjuvant leads to robust $CD4^+$ T cell priming.[301] A strong humoral response is observed in mice immunized with OVA-conjugated anti-Clec12A antibodies, as evidenced by high

specific antibody titers. Furthermore, targeting OVA to Clec12A induces the proliferation of OVA-specific transgenic $CD8^+$ and $CD4^+$ T cells, albeit less effectively compared to Ag targeting to DEC-205 or Clec9A.[299]

1.3.3 Porous nanoparticles

Polymer nanoparticles have attracted attention for their ability to deliver medical drugs as well as vaccine Ags. These nanoparticles are also known to be biodegradable. A great variety of synthetic polymers have been used to prepare nanoparticles, such as poly(d,l-lactide-co-glycolide) (PLG) and poly(d,l-lactic-coglycolic acid) (PLGA), pullulan, alginate, inulin and chitosan. We used porous maltodextrin nanoparticles (NPL), which are known to be potent vaccine formulations with low reactogenicity. These nanoparticles are made of a net of maltodextrin that traps proteins or nucleic acids and negative lipids, in our case the dipalmitoylphosphatidylglycerol (DPPG).[302] Maltodextrin used for nanoparticle preparation is typically produced by starch hydrolysis through an enzymatic process. This polymer of D-glucose contains α-D-glucopyranosyl molecules linked through α-1,4 bounds.[303] It is a polysaccharide generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Maltodextrin nanoparticles (NP+) are produced by chemical synthesis by grafting epichlorohydrin to reticulate the polymer and glycidyltrimethylammonium chloride (GTMA) to confer a positive charge to the particle (Patent US6342226B1, 1998) (Figure 13).

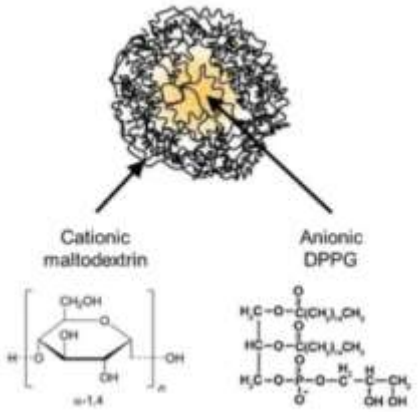


Figure 13. Representation of NPL. The chemical formula of maltodextrin and DPPG are reported.

The safety of the NP+ has been previously investigated. NP+ are not cytotoxic and genotoxic even at high concentrations, therefore NP+ are good and safe candidates for drug delivery.[304] The carrier behavior is modified by the introduction of a negative lipid inside the NP+. Dombu et al. showed that NPL deliver proteins in airway epithelial cells more efficiently than NP+. Moreover, partial endo-lysosomal escape/cytosolic delivery of the protein is observed by OVA-loaded NPL in the same cell model.[305] This property of the NPL can be used to potentially induce MHC I Ag presentation and consecutive cellular response in case of a vaccine formulation. NPL are highly stable carriers, able to associate a high amount of complex proteins. They are effective as vaccine delivery carriers since they induce complete protection against parasitic challenge infection after nasal administration in mice.[306] These carriers are also suitable to deliver lipophilic drugs, such as diminazene.[307] Debin et al. demonstrated that maltodextrin nanoparticles covered by a lipid bi-layer and loaded with HBs Ag and beta-galactosidase were able to induce strong mucosal as well as systemic antibody and cytotoxic T cell

responses, while free Ag was poorly immunogenic.[308] Dimier-Poisson et al. demonstrated that nanoparticles loaded with *Toxoplasma gondii* Ag after intranasal administration were able to induce strong Th1 and Th17 responses and were able to protect mice against an orally administered lethal challenge with wild parasite.[306] Furthermore, Dombu et al. also demonstrated that these nanoparticles were highly endocytosed via the clathrin pathway and highly exocytosed via a cholesterol-dependent pathway, delivering Ag within the cytosol of airway epithelial cells.[305,309] These results might explain the increased immunogenicity observed. [306] Bernocchi et al. further investigated the role of these nanoparticles as potential vaccine delivery systems in airway mucosa, and the different constituents of these NPL were tracked to assess their fate after endocytosis in the mucosa. They first confirmed that the lipid loading into the NP did not vary the characteristics of size and zeta potential of the particles, suggesting the complete lipid incorporation into the maltodextrin structure.[310] They observed similar uptake kinetics for both NPL components, polysaccharide and lipid. This result suggested that the lipids are not released from the nanoparticles in the cells during their endocytosis. This is in contrast to liposomal preparations whose phospholipids were found to be converted to cellular phospholipid after lysosomal degradation.[311,310] They were able to discount any nose-to-brain delivery of these nanoparticles as they were found not to cross the epithelial cells *in vitro* or *in vivo*. Nose-brain passage of nanoparticles and their potential toxicity would prevent further studies for vaccine applications.[312] Furthermore, cytotoxicity and genotoxicity studies were also performed showing that, even at high doses, these NPL were not toxic.[304] The NPL can be loaded with a large amount of different proteins and the formulation is effective to induce humoral, cellular and mucosal responses when administered via the nasal route.[306] Another benefit of the NPL is the simplicity by which we can successfully incorporated other recombinant proteins, such as influenza virus HA in the

nanoparticle. Bernocchi et al. performed bio distribution studies and showed that after 1.5 h the protein administered alone had totally disappeared from the nasal area, while nanoparticle formulated protein was still present after 6 h. These results suggested that NPL stay in the nose and potentially protect the protein from degradation.[310,305]

1.3.4 Liposomes

Lipids are an important building block of many living organisms and they are the main constituents of the cellular membranes.[313] Among the variety of lipids found in living organisms, phospholipids are the most abundant. They are made of a hydrophobic tail, consisting of two fatty acids, linked by a glycerol backbone to a hydrophilic head group, consisting of phosphate and potentially another organic molecule. Taking into account the head group, phospholipids can be classified into 6 categories: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) or phosphatidic acid (PA). PS, PI, PG and PA are negatively charged while PC and PE are neutral but zwitterionic, meaning that those molecules have two or more functional groups, of which at least one has a positive and one has a negative electrical charge and the net charge of the entire molecule is zero. It is possible to chemically modify both the head group and the tail region to synthesize tailored phospholipids.[254] Liposomes are self-assembling particles that consist of a phospholipid bilayer shell with an aqueous core. They can be generated as either unilamellar, consisting of a single phospholipid bilayer, or multilamellar vesicles, that are made of several concentric phospholipid shells separated by layers of water. As a consequence, liposomes can be tailored to incorporate either hydrophilic molecules into the aqueous core or hydrophobic molecules within the phospholipid bilayers.[314] A number of liposome systems have been established for drug delivery and approved for human use, such as Inflexal® V and Epaxal®.[315,316] The liposomes

used in this research project were composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol and1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)-2000] (PEPEGMCC) or POPC, cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (PEMCC) and contained the fusion protein in the core. The fusion protein was also covalently bound to the liposomes using a thiol-maleimide reaction (Figure 14).

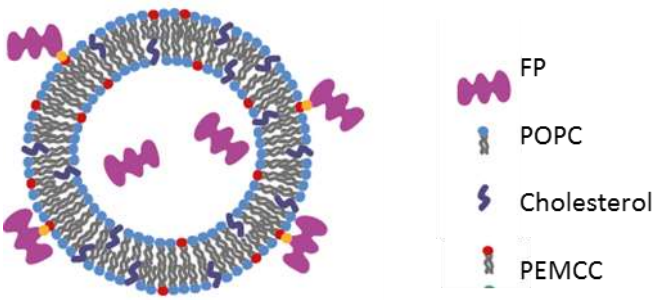


Figure 14. Representation of LNP.

2 AIMS OF THE PROJECT

The general aim of this thesis work was to develop a broadly protective mucosal vaccine against influenza virus infection. The idea was to combine CTA1-3M2e-DD with two different types of nanoparticles to obtain two formulations which could be compared with regard to immunogenicity and protective efficacy. A special focus was given to mechanisms of action and how to achieve an optimal immunogenic nanoparticle.

More specifically, my aims were:

- To test the ability of two different types of nanoparticle formulations for improving immunogenicity and protective capacity of the CTA1-3M2e-DD fusion protein.

- To analyze uptake and processing mechanisms of DCs exposed to the nanoparticles.

- To evaluate the immune priming ability of nanoparticles and assess their protective function against live influenza virus challenge infections.

- To assess the ability of the nanoparticle vaccine to reduce virus transmission from infected to unprotected individuals.

- To determine whether co-incorporated HA could further improve nanoparticle vaccine efficacy.

- To define whether immunizations of neonates could be improved with nanoparticle vaccine formulations.

3 METHODOLOGICAL CONSIDERATIONS

This section provides an overview of the methods used in this study. A detailed description of all the experimental procedures can be found in the attached papers. The work plan to develop this thesis project was divided into four separate, but interrelated, work packages (WP). The first part included the design and development of different constructs exploring the CTA1-DD fusion protein self-adjuvanted vaccine platform. To this end, M2e peptide was incorporated into CTA1-3M3e-DD for the influenza studies, and Eα peptide in CTA1-3Eα-DD for the studies on DC presentation (WP1). The fusion proteins were then formulated into nanoparticles and characterized (WP2). A third part of the project addressed the uptake of the nanoparticle vaccine candidates (WP3). The final part assessed the immunogenicity of the nanoparticle formulations after mucosal immunizations and the protective capacity of nanoparticle-based vaccines using the live virus challenge models in mice (WP4).

3.1 WP1: Fusion protein construction

The CTA1-DD fusion protein with enzymatic activity and CTA1(R9K)-DD, the enzymatically inactive mutant, carrying the M2e or Eα peptides were produced and expressed in *E. coli* DH5 cells, as described.[317] The fusion proteins carried three tandem repeats of the peptides. For a complete list of peptides inserted in CTA1-DD fusion protein see Table 3. The molecular weight of the fusion proteins was estimated to be 45 kDa.

Fusion protein	Peptide	Sequence	Length	Ref.
CTA1-3M3e-DD	M2e	SLLTEVETPIRNEWGSRSNDSSD	23aa	[318]
CTA1-3Eα-DD	Eα	ASFEAQGALANIAVDKA	17aa	[319]

Table 3. List of peptides included in different CTA1-DD fusion proteins.

We used the Gate Assembly strategy to incorporate the selected peptides into the CTA1-DD DNA sequence.[320] Briefly, this technique allows multiple inserts to be assembled into a vector backbone using only the simultaneous activities of a single Type IIS restriction endonuclease (REase) and T4 DNA ligase. This technique exploits the ability of REase to cleave DNA outside of the recognition sequence. The inserts and cloning vectors are designed to place the REase recognition site distal to the cleavage site, such that the REase can remove the recognition sequence from the assembly (Figure 15). One advantage of this technique is that the fragment-specific sequence of the overhangs allows for an orderly assembly of multiple fragments simultaneously. Moreover, since the restriction site is eliminated from the ligated product, digestion and ligation can be carried out concomitantly.

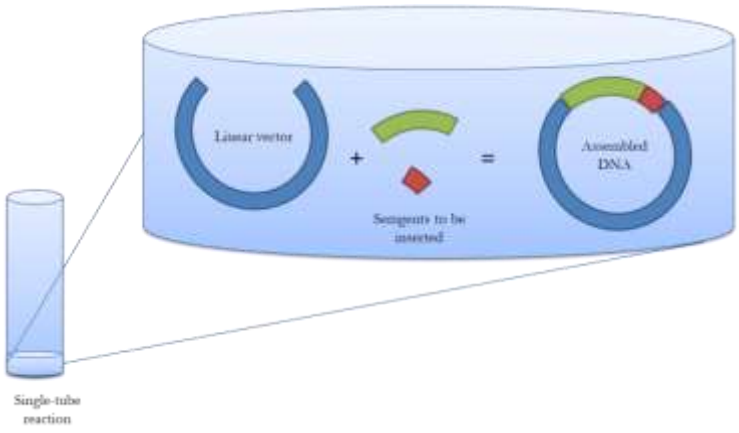


Figure 15. Golden Gate Assembly cloning strategy. In a single reaction tube the backbone plasmid vector and the plasmid containing the insert are mixed with an endonuclease and a ligase enzyme.

E.coli DH5 cells were transformed with the DNA vector of interest and harvested by centrifugation. The fusion proteins, produced as inclusion bodies, were washed before extraction by treatment with 8M urea. After refolding of the proteins by slowly diluting them in Tris-HCl, the fusion proteins were purified in two steps, by ion exchange (Figure 16) and size exclusion liquid chromatography (Figure 17).

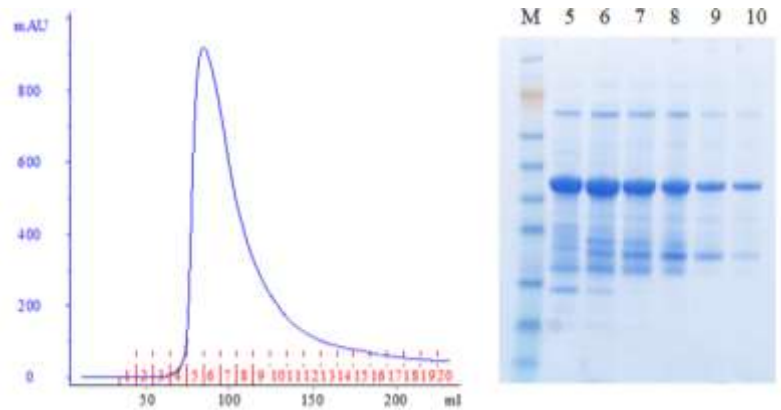


Figure 16. Ion exchange liquid chromatography. Plot illustrating protein separation with a typical ion exchange column (left panel). Protein analysis by SDS-PAGE (right panel).

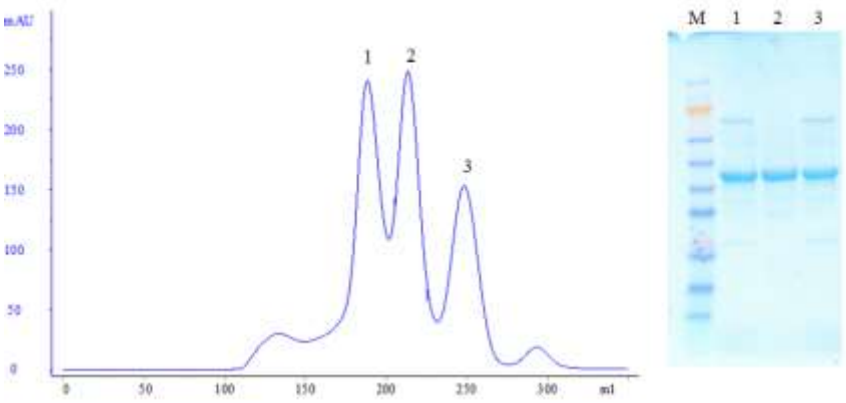
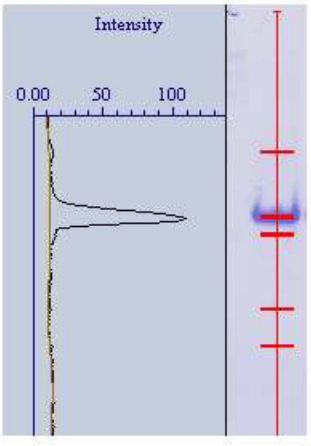


Figure 17. Size exclusion liquid chromatography. Plot illustrating protein separation with size exclusion (left panel). Protein analysis by SDS-PAGE (right panel).

After concentration and sterile filtration, the purified fusion proteins were stored at -80°C until use. Proteins were analyzed for their purity by SDS-PAGE (Figure 18) and concentrations were determined using BCA assay.



Band #	Peak Int	Trace Int x mm	Relative Qty
1 - 1	97.71	220.241	93.9

Figure 18. Purity determination. Protein purity analysis by Western blotting and Image J software.

The fusion proteins were routinely tested for the presence of endotoxin by the end-point chromogenic limulus amebocyte lysate method.[321] The fusion proteins were used in experimental models only if the endotoxin-levels were below 100 endotoxin units/mg (EU/mg). ADP-ribosyltransferase enzymatic activity was tested using the NAD:agmatine assay, as described.[322]

3.2 WP2: Nanoparticle preparation

To improve vaccine efficacy with an enhanced stability for mucosal administration, we combined the fusion proteins with two different types of nanoparticles. To this end, several possibilities were explored. We tested encapsulating as well as surface exposing the fusion protein on the nanoparticle or combining the two. By encapsulating antigenic material, nanoparticles protect against Ag-degradation, which has proven important for oral vaccine administration. Of note, conjugation of the fusion proteins to the nanoparticles can allow for processing and presentation of the epitopes in the same way as it would be presented by the pathogen.

3.2.1 Porous maltodextrin nanoparticles (NPL)

To produce polysaccharide nanoparticles maltodextrin was usually dissolved in an organic solvent (sodium hydroxide, ethyl acetate or methylene chloride) followed by the addition of a mixture of epichlorohydrin, glycidyltrimethylammonium chloride and the fusion protein, which was then vortexed to obtain a primary emulsion.[302] The water-in-oil-in-water emulsion was then formed with the addition of an emulsifying agent (polyvinyl alcohol or polyvinyl pyrrolidine). This

resulted in that the polymer precipitated around the fusion protein. Lyophilized nanoparticles were dissolved in water and a lipid (DPPG: 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol) was loaded into the nanoparticles. The complex was then left to allow solvent evaporation, which was followed by a drying step to prevent degradation of the polymer due to water-catalyzed ester hydrolysis (Figure 19). This way we obtained a formulation fusion protein:NPL at different mass ratio (1:0,5,1:3,1:5,1:10).

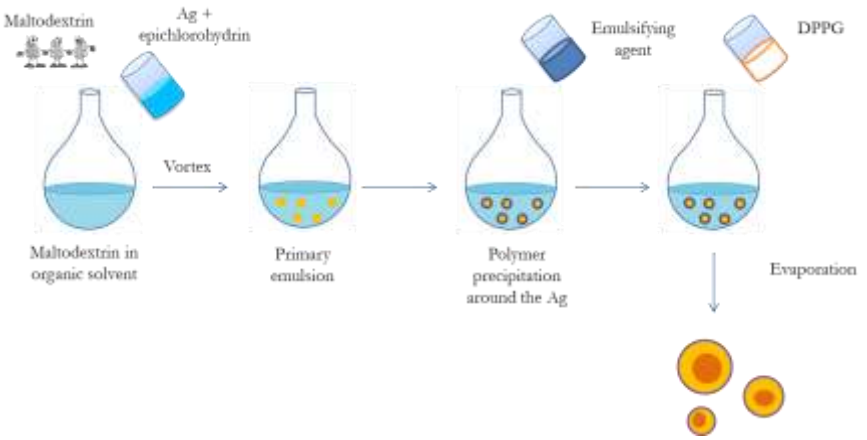


Figure 19. Porous maltodextrin nanoparticles preparation.

The size and the zeta potential of fusion proteins and NPL were determined in water by dynamic light scattering. The analysis of fusion protein association to NPL was performed by native polyacrylamide gel electrophoresis (PAGE). The gel was stained by the silver nitrate method to detect unbound fusion proteins. Moreover, the stability of the fusion protein and of the nanoparticles was evaluated over 3 months, under standard (4°C) or accelerated (40°C) conditions, or after 12 months at 4°C.

3.2.2 Liposomes (LNP)

Methods for producing liposomes typically involve a reverse phase evaporation process to dissolve the phospholipids (monophosphoryl lipid A or phosphatidylcholine) in an organic solvent (chloroform or methanol). In particular, we used 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethy-leneglycol)-2000] (PEPEGMCC) or POPC, cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimido-methyl) cyclohexane-carboxamide] (PEMCC) in methanol/chloroform. Water was then added, along with the fusion protein, and the solvent was evaporated. The solution was then extruded 11 times through two 100 nm nucleopore track-etched polycarbonate membranes (Figure 20). The fusion protein was covalently bound to the liposomes using a thiol-maleimide reaction.

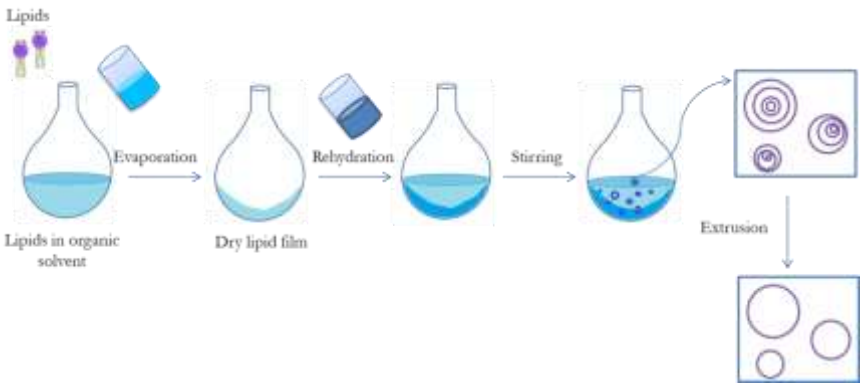


Figure 20. Liposome preparation.

The size distributions of the lipid nanoparticles were determined using nanoparticle tracking analysis (NTA). NTA measurements were performed over a period of more than 100 days in order to assess the stability of the lipid nanoparticles. Liposome concentration and the zeta potentials were measured. In order to monitor the lamellarity of the

particles cryogenic transmission electron microscopy (Cryo-TEM) was performed. The fusion protein content was determined using the CBQCA Protein Quantitation Kit. In order to assess the amount of material lost during vesicle production, a liposome was assumed to be a spherical double lipid layer of the average diameter determined by NTA containing lipids with a footprint of 0.68 nm^2.

3.3 WP3: Antigen presentation assessment

To study nanoparticle uptake and presentation by APCs and the priming ability of $CD4^+$ T cells, we sequentially screened and compared fusion proteins and nanoparticles. First, the immunogenicity was assessed *in vitro* and, thereafter, we performed *in vivo* experiments to determine the ability of DCs to prime $CD4^+$ T cells in draining lymph nodes.

3.3.1 *In vitro* assays

We used two different screening assays. The first one employed H-2d-restricted B cells (A-20) and a $CD4^+$ T cell hybridoma specific for M2e complexed with MHC II that upon TCR recognition produced IL-2.[213] The latter was assessed by an IL-2 dependent CTLL-2 proliferation assay. Limiting dilutions (starting from 0,2uM) of M2e peptide, CTA1-3M2e-DD or nanoparticles that carried CTA1-3M2e-DD were tested. The IL-2 content was assessed by [3H]-thymidine uptake in proliferating CTLL-2 cells cultured for 24h.

The second assay employed the D1 cell line, a long-term growth factor-dependent immature myeloid ($CD11b^+$, $CD8\alpha^-$) DC line of splenic origin derived from a female C57BL/6 mouse (Figure 21).[323]

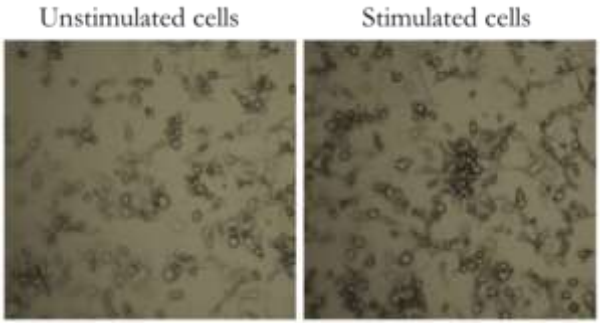


Figure 21. D1 cells after stimulation tend to aggregate in clusters.

In this assay we determined the nanoparticle processing ability of DCs and detected Eα peptide on I-A^b MHC II molecules using the Y-Ae antibody, specific for the complex (Figure 22).[319] The level of MHC II-Eα complexes can be assessed by FACS after labeling with the Y-Ae antibody. Thus, the Y-Ae antibody detects the same epitope-MHC complex as the T cell receptor does and, in this way it could be used as proxy for immunogenicity. The CTA1-3Eα-DD was tested alone or incorporated in nanoparticles. We determined the ability of D1 cells to present a wide range of fusion protein and nanoparticle concentrations and correlated the MFI with the expression level of MHC II using a specific monoclonal antibody (mAb) for I-A^b.

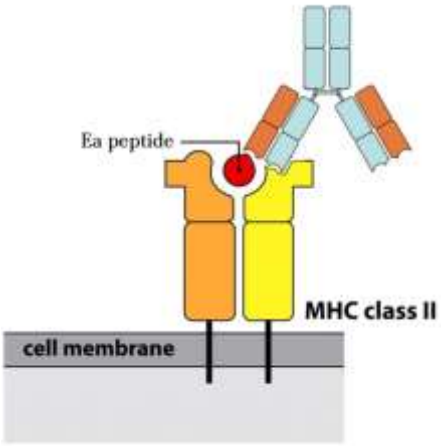


Figure 22. Ea peptide in the MHCII groove is recognized by a monoclonal antibody, Y-Ae.

Also other markers reflecting DC activation were assessed by FACS using specific mAbs against CD80, CD83 and CD86. These experiments were complemented with microscopic analysis of fusion protein distribution in subcellular compartments. We labeled the fusion protein with Alexa488 fluorochrome and exposed D1 cells in Cell Culture Chamber Slides for the fusion protein or nanoparticles. Endosomal and lysosomal compartments were labeled with Mabs specific for EEA-1 and LAMP1 respectively, and the nuclei were visualized with DAPI. Confocal microscopy was performed at the Centre for Cellular Imaging.

3.3.2 Eα peptide tracking on DCs and CD4$^+$ T cells priming

In order to evaluate which DC subtype took up the nanoparticle and processed the fusion protein *in vivo* we used B6.Cg-Tg(TCRα,TCRβ)3Ayr/J mice that were TCR transgenic for the Eα complexed to H2-A^b (I-A^b). This mouse lacks naturally occurring Eα peptide as the coding gene in C57BL/6J mice is incapable of expressing a protein product. Flow cytometric analysis showed 90% and 95% of CD4$^+$ splenocytes expressing TCRα-V2 and TCRβ-V6, respectively.[323] Mice were given a single intranasal immunization with 50ug of fusion protein in the form of soluble protein or incorporated into nanoparticles and the mLN were collected after 24 hours. Migratory (MHC II^{high}, CD11c$^+$) and

resident (MHC II^{low}, $CD11c^{+}$) DCs were identified and the presence of Y-Ae positive cells was analyzed by FACS. A population of Y-Ae^{+} cells was identified and quantified in the migratory DCs population in immunized mice (Figure 23).

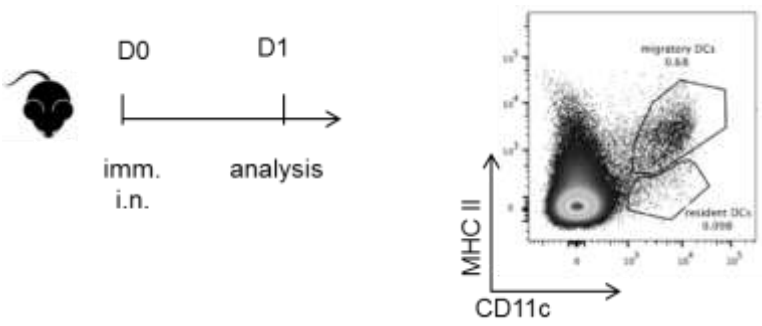


Figure 23. Migratory and resident DCs tracking. Experiment design (left panel) and FACS plot to gate for DCs populations (right panel).

A confounding element in these experiments is the much higher dose (10-fold) of fusion protein needed as compared to the normal immunization dose. This was necessary as we failed to detect DCs with peptide when we used lower doses.

Next, we assessed the priming ability *in vivo*. We used an adoptive transfer model where TCR transgenic $CD4^{+}$ T cells were injected into wild-type C57BL/6 mice. These $CD4^{+}$ T cells were labeled with CFSE to allow for detection of proliferating cells *in vivo*. After uptake of nanoparticles, migratory DCs were found in the draining lymph node, where $CD4^{+}$ T cell priming was assessed. At different time points after transfer, mLNs were collected and TCRα-$V2^{+}$TCRβ-$V6^{+}CD4^{+}$ T cells were analyzed for the level of CFSE labeling, used as an indicator of cell divisions (Figure 24).

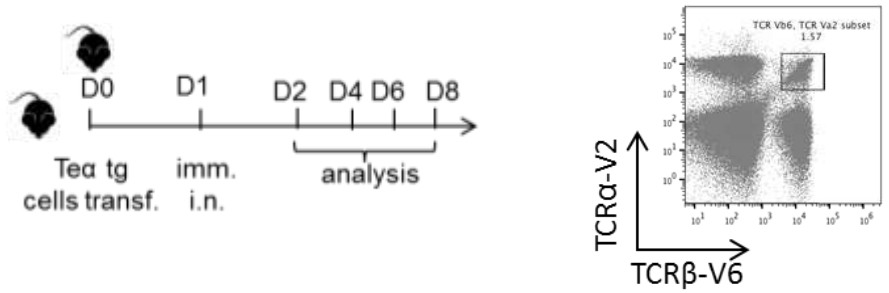


Figure 24. Adoptive transfer protocol. Experiment design (left panel) and FACS plot to gate for TCRα,TCRβ $CD4^+$ T cells (right panel).

A modification of this protocol was then used to determine for how long migratory DCs could prime $CD4^+$ T cells in the mLN. To do this, adoptively transferred C57BL/6 mice were immunized with the CTA1-3Eα-DD and at different time points, thereafter, mice were injected with $2x10^6$ B6.Cg-Tg(TCRα,TCRβ)3Ayr/J CFSE-labeled $CD4^+$ T cells and after 4 days their proliferation was determined (Figure 25).

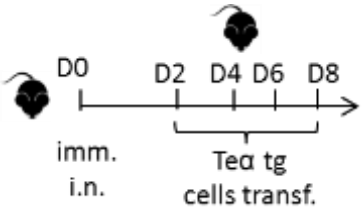


Figure 25. Modification of the adoptive transfer protocol.

3.4 WP4: Mechanisms of action

By comparing soluble fusion protein to nanoparticle bound fusion protein, we could determine the relative immunogenicity of the different formulations. Following the last immunization spleens, mLN, and lungs were harvested and recall responses were assessed *in vitro*. We then

assessed the level of IFN-y and IL-17 in culture supernatants by ELISA and ELISPOT. By FACS we determined the presence of M2e-specific $CD4^+$ T cells in the lungs using an M2e-tetramer specifically designed for these studies. To evaluate whether $CD4^+$ T cells specific for M2e contributed to protection, we depleted all $CD4^+$ T cells using 1mg/mL *nVivoMAb* anti-mouse CD4 antibody clone GK1.5 i.p. 3 times 24h apart prior to the virus challenge infection. We also assayed for Ag-specific antibodies in broncheolaveolar lavage (BAL) and serum.

Protection against infection was evaluated against a live virus of different strains. A potentially lethal dose of 4 LD50, corresponding to 2.5×10^3 TCID 50) of mouse adapted reassortant influenza A H3N2 virus strains X47 (A/Victoria/3/75 (H3N2) with A/Puerto Rico/8/34(H1N1)) or A/Puerto Rico/8/34 (H1N1) was administrated intranasally to mice that had been lightly anesthetized by isofluoran solution. Mortality was monitored on a daily basis for two weeks after challenge. Morbidity was followed at one-day intervals by monitoring body weight and mice were sacrificed if they lost more than 30% of their weight. Next, we tested the ability of the combined vector to impair virus transmission. We used the recently established mouse model with DBA/2 mice as contact mice (C).[324] Following a challenge infection with Udorn virus (H3N2), immunized and unimmunized Balb/c mice (index mice, I) were co-housed with the contact mice for 4 days and virus transmission was determined by measuring viral titers in the snouts and lungs of the index mice (Figure 26).

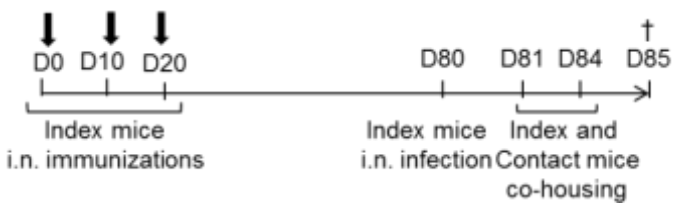


Figure 26. Transmission experiment protocol.

4 RESULTS

The fusion protein CTA1-3M2e-DD has previously been found to stimulate strong protective immunity against a heterosubtypic influenza A virus strain challenge and, therefore, was considered a candidate for a broadly protective influenza vaccine.[213] It has repeatedly been found that the use of particulate Ags can be more effective than soluble proteins at stimulating strong immune responses and affording long-term protection.[302, 306, 325-327,328] To improve the immunogenicity and stability of our vaccine candidate we speculated that incorporating the fusion protein into nanoparticles could be advantageous. [254] Two different nanoparticle strategies were tested by i.n. immunizations, the first one based on a positively charged maltodextrin nanoparticle and the second on a negatively charged liposome. We also attempted oral immunizations with the aim of developing a vaccine for neonates. The results from the studies are presented in Paper I-III and will be summarized in this chapter.

4.1 Paper I - Porous nanoparticles with incorporated adjuvant and recombinant hemagglutinin

The first nanoparticle formulation that we successfully used for mucosal immunizations consisted of porous maltodextrin particles (NPL).[302] The porous NPL technology has been successfully used for several i.n. vaccine formulations in the past, including a vaccine candidate against toxoplasma infection.[329] An advantage of these particles is that they allow

multiple components to be incorporated into the particle.[306] We manufactured the NPL and characterized their physico-chemical properties. We evaluated their ability to enhance the immunogenicity of the CTA1-3M2e-DD fusion protein (FPM2e) and, thereafter, we attempted to combine the fusion protein with recombinant HA into one NPL. Combining the CTA1-3M2e-DD with HA from influenza virus and the NPL formulation we achieved an even more potent subcomponent mucosal vaccine formulation. Several other studies support the concept of multiple influenza Ags encapsulated into nanoparticles as a promising way forward for a broadly protective influenza vaccine.[330,331] Whereas many previous studies have reported on promising mucosal vaccine candidates against influenza, this is the first to describe the combination of an enzyme-active adjuvant system incorporated into nanoparticles.[332,333-334]

4.1.1 Dendritic cells effectively uptake the vaccine candidate

Since little is known about DC uptake and presentation of Ags delivered with these NPL, we initially focused on the DCs.[335] To analyze Ag uptake and processing, CTA1-3Eα-DD was formulated into NPL (FPEα:NPL) in order to detect the Eα peptide when bound to MHC II surface molecules on DCs.[319] Stimulating D1 cells with the different constructs, we could observe a higher expression of MHC II and Eα-MHC II on DCs exposed to the combined vector as opposed to when the fusion protein was used alone. B6.Cg-Tg(TCRα,TCRβ)3Ayr/J mice, which host TCR transgenic $CD4^+$ T cells that recognize the Eα peptide bound to MHC II, were instrumental to determine whether the combined formulation was taken up by DCs *in vivo*. Eα peptide was found in 20% of the migratory DCs while resident DCs appeared to be less involved in the vaccine uptake. In an adoptive transfer experiment B6.Cg-Tg(TCRα,TCRβ)3Ayr/J $CD4^+$ T cells were injected into C57BL/6 immunized mice and their expansion was followed after i.n

immunization. $CD4^+$ T cells in the mLN were proliferating slower in mice immunized with the NPL vaccine but the expansion was sustained until at least day 12 after immunization, when proliferation to the fusion protein only was minimal. While the effect *in vitro* indicated a dramatic improvement of peptide expression in exposed DCs, the *in vivo* expression in migratory DC was comparable between fusion protein alone and formulated into NPL. NPL formulations are retained in the nasal mucosa longer than the soluble Ag and this leads to a slower and more prolonged priming of specific $CD4^+$ T cells in the mLN. Also earlier studies have observed a depot-effect and retention of $CD4^+$ T cell priming in draining lymph nodes when nanoparticle formulations were used.[336] Therefore, it may be possible to further improve the performance of the vector by altering the chemical composition of the NPL or by adding chitosan or some known component with an effect on the penetration of the mucosal barrier.[337,338,339,340]

4.1.2 Enhanced immunogenicity and protection against virus transmission

To determine the immunogenicity of the formulated vaccine we immunized Balb/c mice i.n. and we found that the combined NPL vector stimulated significant responses. At lower doses of Ag, the NPLs were more effective than the soluble fusion protein. Moreover, the ADP-ribosylating activity of the CTA1-enzyme was a necessary requirement for the strong enhancing effect of the fusion protein on immunogenicity. This has previously been documented.[341] We assessed the protective efficacy against a challenge with X47 virus strain, a mouse adapted reassortant A/Victoria/3/75 (H3N2) virus strain.[342-343] Infected mice were monitored for weight loss and survival for 15 days post-infection. We found that protection was higher in mice immunized with the formulated vector. The protective effect was associated with a strong $CD4^+$ T cell priming effect for IFN-γ and IL-17 production, which are

the cardinal features of strong heterosubtypic protection in the mouse model of influenza infection.[344,345,346,347,348] We could identify the presence of lung resident M2e tetramer-specific $CD4^+$ T cells, which have a very important role for heterosubtypic protection.[213] Strong M2e-specific antibody responses in serum and BAL were found. This enhancing effect is what we have seen with CTA1-DD adjuvant in many other studies.[144,244,142] We also tested the ability of the combined vector to impair virus transmission between animals.[324] Following a challenge infection with Udorn virus (H3N2), immunized and unimmunized Balb/c mice (index mice) were co-housed with the contact mice and virus transmission was assessed. Lower virus titres were found in the snouts of the contact mice co-housed with index mice immunized with the formulated vaccine indicating that the nanoparticle formulation helped reducing viral transmission among animals.

4.1.3 Combinations of fusion protein and recombinant HA provides increased protection of the fusion protein/nanoparticle vector

To improve the protective ability of the NPLs, we incorporated recombinant hemagglutinin (A/H1N1/PR8/34) with the CTA1-3M2e-DD adjuvant. We found that mice immunized i.n. with the combined vector containing HA were fully protected against a challenge infection with the highly virulent PR8 virus (A/Puerto Rico/8/34 (H1N1). HA-specific IgG serum antibody responses were substantially augmented and mice were also protected against X47 virus infection. It was clear that the immunogenicity of the incorporated HA greatly benefitted from the adjuvant enhancing effects as anti-HA serum IgG titers were higher. To assess whether the antibodies were able to neutralize the virus, we pre-incubated mice sera with PR8-GFP virus and used it to infect MDCK cells. Whereas 80% of MDCK cells were infected, immune sera of mice immunized with HA:NPL, FPM2e:NPL and HA:FPM2e:NPL were

strongly neutralizing as opposed to serum from fusion protein alone immunized mice, which were only partially neutralizing (Figure 27).

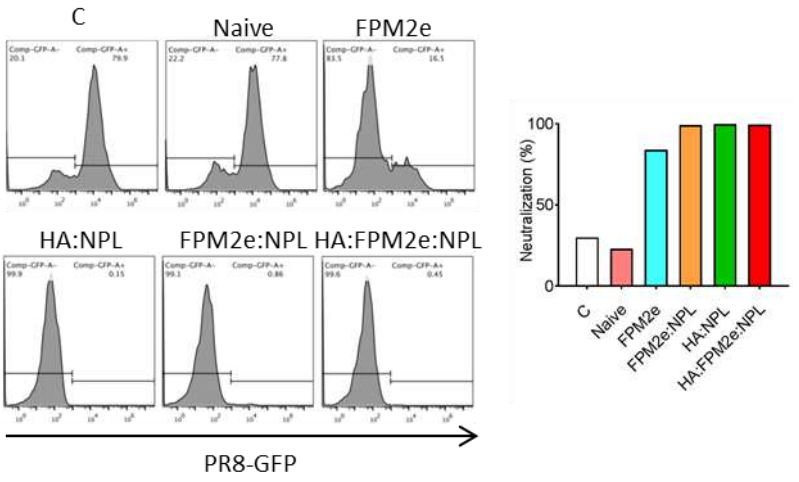


Figure 27. Neutralization assay. Histograms for GFP-PR8-infected MDCK (left panel) and % of neutralization of the infection (right panel).

Importantly, the immunogenicity and protective capacity of the combined vector was critically dependent on the enzymatic activity of CTA1. Interestingly, excellent protection was achieved also with NPLs where HA and the fusion protein were formulated in separate particles. However, the augmenting effect on anti-HA IgG serum antibodies was not seen when the fusion protein and HA were provided in separate NPLs, suggesting that this effect required physical contact between HA and the FPM2e. By contrast, anti-HA-specific cell-mediated immunity was enhanced irrespective of if HA and the fusion protein were in separate or in a single particle. This result is in agreement with a direct effect of the CTA1-3M2e-DD on the FDCs in the GC, which could only work if expanding HA-specific B cells were recruited to CTA1-3M2e-DD exposed FDCs.[349,337] Indeed, we have recently found that CTA1-DD adjuvant has a direct effect on FDCs by up-regulating gene transcription

and, in particular, the CXCL13 gene. CXCL13 is the main chemokine to attract activated B cells into the GC.[350] The enhanced $CD4^+$ T cell priming, on the other hand, would depend more on improved DC-function, which doesn't appear to require the close physical contact between fusion protein and the NPL.

4.2 Paper II – Liposomes-based vaccine

Different lipid-based vaccine candidates against many different pathogens have been developed during the past 20 years, but no commercially available vaccine based on this technology exists yet.[351] We have previously combined CTA1-3M2e-DD with immune stimulating complexes (ISCOMs) to achieve a particulate vaccine formulation, with excellent and improved immune stimulating properties when given intranasally.[317] However, while ISCOMs are stable, protein-containing cage-like structures composed of cholesterol, phospholipids and saponins from the *Quillaja saponaria Molina* tree, they have limitations in their Ag loading capacity and may also be toxic due to the immunomodulating saponins.[352,353,354] Therefore, to get a more versatile and dynamic particle, we explored the liposome technology. We undertook a series of experiments to characterize the physico-chemical properties of the liposomes and we evaluated their influence on Ag-uptake by DC, the effect on T-cell priming, and antibody-stimulating ability. We also tested their ability to protect against a live challenge infection with influenza homo- or heterosubtypic virus strains. Moreover, we investigated if PEGylation had a positive effect on the nanoparticle immunogenicity. We hypothesized that adding PEG spacers to the fusion proteins would increase coating efficiency and make more protein accessible to the

immune system. However, PEGylation has been previously been used to enhance the particle retention time at mucosal membranes or used to avoid adsorption of proteins from the circulation. It was proposed that the formation of a corona around the nanoparticle would mask the surface of particles and increase retention.[355,356,281] Noteworthy, high molecular weight polymers (>PEG5000) are preferentially mucoadhesive, while molecular lower weights (for example PEG2000, the one used in this work) appear to diffuse better through the mucus layer.[357,358]

4.2.1 Liposome vectors improve antigen uptake, presentation and T cell priming

LNPs were made with the fusion protein bound to the surface as well as incorporated inside the particle. Earlier studies reported that CTB was effective when bound to the surface of the liposome.[359] We then evaluated the interaction between the liposomes and APCs using two different *in vitro* models for Ag presentation as described in the M&M section of my thesis. We observed in both models, at early time points, enhanced expression of pMHC-II complexes on the APCs, but after 2h of peptide expression was similar.[360] Moreover, the expression level of MHC II and CD86 molecules was higher on DCs exposed to nanoparticles. Thus, the combined LNP vector was superior to soluble protein alone for uptake, processing and MHC II presentation.

Confocal microscopy on D1 cells following incubation with nanoparticles showed that the liposomes effectively entered some target cells while the soluble Ag was taken up into the APCs more evenly in a dose–dependent fashion. The soluble Ag can be visualized in the cell cytoplasm after a short incubation time (Figure 28).

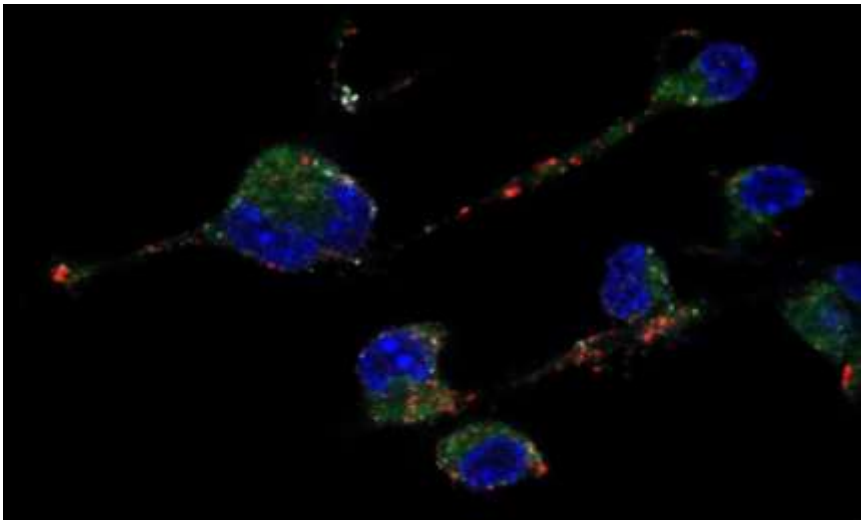


Figure 28. Confocal microscopy on stimulated D1 cells. Nuclei (blue), construct (green), EEA-1 (red), LAMP-1 (white) are shown.

Both the soluble fusion protein and that bound by liposomes were found within the early endosome compartment. Whereas liposomes targeted fewer cells, the soluble fusion protein was taken up by a larger number of cells (Figure 29). Differential modes of intracellular trafficking into endosomal or lysosomal compartments have been found to directly influence the efficiency and mode of T cell priming.[87]

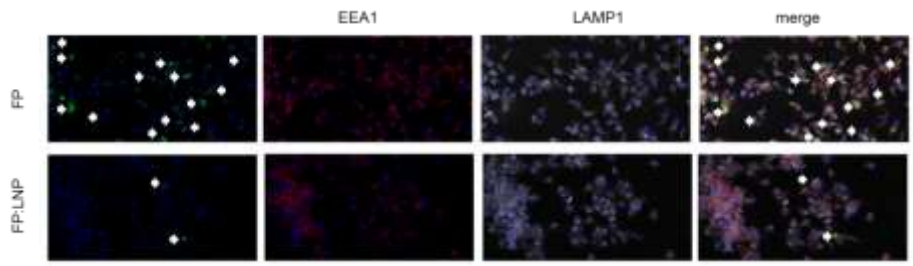


Figure 29. Fluorescent microscopy on stimulated D1 cells. Nuclei (blue), construct (green), EEA-1 (red), LAMP-1 (white) are shown.

We analyzed the T cell priming efficiency *in vivo* by using an adoptive transfer model with TCR transgenic mice injected into C57BL/6 mice. We observed effective T cell proliferation initiated earlier by the soluble fusion protein than by the LNP formulation. Thus, the liposome vector primed $CD4^+$ T cells at a later time point but T cell proliferation continued until at least day 8, when proliferation to the soluble protein had declined significantly. Moreover, the $CD4^+$ T cell priming ability in the mLN was maintained for long with both soluble protein and LNP immunizations (at least 8 days).

4.2.2 Local immunity is enhanced by combined LNP vaccine vectors

Immunogenicity of the combined LNP vector was evaluated *in vivo* following three i.n. immunizations in Balb/c mice and specific M2e-IgG antibodies in serum were determined. High antibody titers were recorded already after two immunizations with LNP vectors, while soluble fusion protein was less effective. However, after three immunizations the specific IgG antibody levels in serum and the recall cytokine and proliferative response to M2e-peptide in whole splenocytes exhibited comparable $CD4^+$ T cell priming efficiency of both regimens. As seen before, the enhancing effects were strictly dependent on the ADP-ribosylating ability of the CTA1-moiety. Most importantly, whereas the systemic enhancing effects of fusion protein and LNP vectors were comparable, the local immune response was significantly stronger in LNP immunized mice. The mucosal anti-M2e IgA titers in BAL were stronger, and the resident M2e-tetramer specific $CD4^+$ T cell response in the lung was dramatically improved.

The question of whether PEGylation was advantageous or not was clearly answered by these experiments. We found significantly weaker responses in PEGylated LNP immunized mice than in non-PEGylated

LNP immunized mice. Maybe, PEGylation provided a steric hindrance for processing of M2e epitopes.[361] Of note, though, PEGylation of proteins and peptides have previously been found to increase mucus-penetrance, which could have increased immunogenicity.[362,363] In our case, we failed to observe a positive effect of PEGylation, but it could be possible that with a different lipid composition PEGylation could work in favor of an improved mucosal immune response.

To assess the protective potential of the combined vector, we challenged i.n. immunized mice with reassortant influenza A H3N2 virus strains X47 (A/Victoria/3/75 (H3N2) with A/Puerto Rico/8/34(H1N1)) or the heterosubtypic PR8 A/Puerto Rico/8/34(H1N1) influenza virus strains. Immunized and challenged mice demonstrated 80 to 100% survival after challenge with the X47 virus strain. Most strikingly, only LNP vectors induced significant protection against a lethal challenge dose with the heterosubtypic PR8 virus strain. This was also reflected in the less severe lung tissue damage observed macroscopically and, in fact, at 7 days post infection no gross lung pathology was observed in contrast to fusion protein only immunized mice that succumbed to infection and exhibited hemorrhagic pneumonia. Protection was clearly correlated to an enhanced frequency of lung resident M2e-specific $CD4^+$ T cells and local IgA antibodies. This is in agreement with our previous study in congenic Balb/c and Balb/b mice, which showed strong protection and M2e-spepcific $CD4^+$ T cells only in Balb/c mice.[213] To confirm the importance of $CD4^+$ T cells for protection, we depleted $CD4^+$ T cells in fully immunized mice just prior to a challenge infection and found that this lead to that mice succumbed the infection, despite having comparable anti-M2e IgG2a antibodies to those of untreated immunized mice (Figure 30).

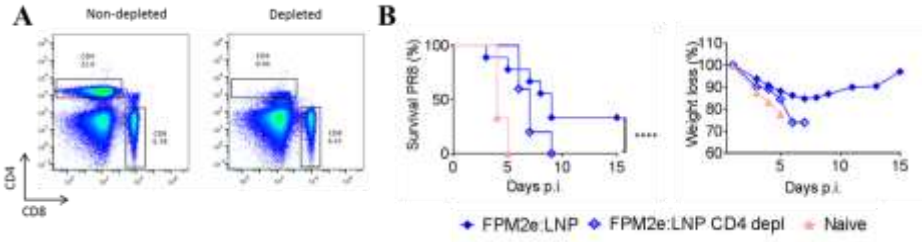


Figure 30. Depletion experiment. A) FACS plot showing successful depletion of $CD4^+$ T cells in lungs. B) Survival and weight loss were monitored. Percent of surviving mice (left panel) and body weight loss (right panel) following a challenge infection with 4×LD50 of PR8 virus strain.

When we determined the lung viral titers in the immunized mice we, unexpectedly, found that both groups of immunized mice had similar viral loads. Thus, despite differences in survival after $CD4^+$T cell depletion, the viral titers were comparable (Figure 31). This finding could indicate that the LNP immunization stimulated $CD4^+$ T cells that provided protection against tissue destruction and preserved lung function, suggesting that an anti-inflammatory component in immunized mice was decisive for survival.

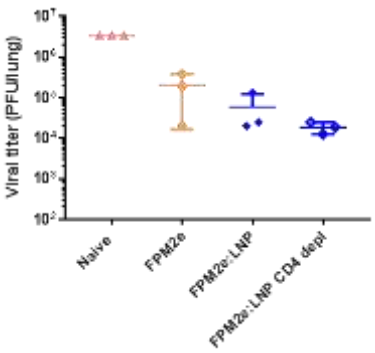


Figure 31. Viral titer (PFU/lung) in lungs of immunized and challenged mice.

4.3 Paper III - Development of a mucosal vaccine for neonates and young infants

Protection against influenza virus in newborns and infants is achieved through transplacental transfer of maternal antibodies to the fetus. The duration of this protection is limited to a few months after birth and it is dependent on whether the mother has developed immunity to the specific virus strain that the child is exposed to. Therefore, much effort has been invested into vaccine development for neonates. However, the neonatal immune system is immature, which poses a problem for vaccine development. In fact, neonatal vaccination has been a much proposed solution to global health interventions in the infectious disease field. The lack of effective vaccines contributes greatly to an increased risk of morbidity and mortality among neonates and young infants. A hallmark of young infants is the poor ability to develop GC reactions and to develop memory B cells and raise isotype-switched antibodies.[364,365] For example, the trivalent influenza vaccine (TIV) showed limited protective efficacy in infants between 6 and 25 months of age, while in infants younger than 6 months no or very poor effects were shown. Moreover, this was associated with significant adverse reactions.[366,367,368,369] It is commonly agreed that more efficacious, but also safer, adjuvants would dramatically improve our chances to develop neonatal vaccines. Therefore, the search for vaccine adjuvants that can stimulate maturation of the neonatal immune response to become more adult-like is much warranted. Recent studies compared various adjuvants for enhanced GC and antibody responses in neonatal mice and found that C-type lectin receptors (CLR) agonists were more potent than TLR-based adjuvants in circumventing the limitations of the neonatal B cell response.[370] Moreover, a mutated version of the heat-labile enterotoxin of *E.coli* (LT-K63) has previously been demonstrated to enhance the GC reaction by a

mechanism that was proposed to involve FDCs, but no experimental support was provided in that study.[371]

In the third paper, we asked whether CTA1-DD combined with M2e peptide in a nanoparticle formulation could work as an effective mucosal vaccine in neonates. We specifically focused on the induction of the GC reaction and the effect on the immune response against influenza infections. CTA1-DD is able to significantly enhance GC reactions, promote development of memory B cells and long-lived plasma cells, as well as stimulate a strong and balanced $CD4^+$ T cell response in adult mice.[143,372,373] A key mechanism of the CTA1-DD adjuvant is the activation of complement enabling it to have a direct binding via the complement receptors 1 and 2 (CR1,CD35 / CR2,CD21) to FDCs.[350] This way, it modulated the FDC function and augmented the GC reaction. Hence, we investigated whether also in neonatal mice we could observe a direct effect of CTA1-DD on FDC maturation and function. If so, we explored whether this effect was sufficient to enhance GC development and augment antibody-, as well as $CD4^+$ T cell, responses leading to immune protection against influenza.

4.3.1 CTA1-DD accelerates FDCs network maturation in B cell follicles in neonates

In a complementary set of experiments we addressed whether CTA1-DD could exert a maturational effect on the FDC following immunization. We found strong evidence for a direct modulating effect of the adjuvant on FDC functions (Schussek et al., manuscript in preparation). The mechanism involved an augmenting effect on gene transcription, in particular the CXCL13 encoding gene, in FDCs following CTA1-DD administration. This effect was ADP-ribosyltransferase-dependent and exclusive for FDCs and required CR2 (CD21) expression. A gene-reporter mouse was developed in which GFP was expressed under the

CD21Cre-promotor. Using this model to better identify FDCs we could investigate lymphoid tissues in neonatal mice for the presence and status of the FDC networks. We identified no FDCs in the spleen or peripheral lymph nodes until 17 days of age. After subcutaneous (s.c.) administration of CTA1-DD adjuvant to neonates, however, we found newly developed FDC networks in 10% of the B cell follicles. Thus, already at 7 days of age FDC networks could be stimulated to mature by CTA1-DD treatment. Thus, CTA1-DD adjuvant exerted a maturational effect on the FDC network in neonatal mice.

4.3.2 Tfh and GC B cell responses are greatly promoted by CTA1-DD adjuvant treatment

In adults, Tfh cell responses directly translate into GC induction and strong antibody responses.[370] Also, Tfh cells have a critical role in the impaired development of GC reactions in neonates.[374,375] To assess whether the FDC-maturational effect of CTA1-DD affected GC functions in neonatal mice, we analyzed lymph node GC development after 10 days following immunization with CTA1-DD. We found that the Tfh responses in neonatal mice were significantly enhanced in the presence of the adjuvant and that we observed an enhanced frequency of GC B cells in both adult and neonatal mice. Furthermore, we found that specific isotype-switched antibodies in serum were significantly enhanced in neonatal mice already at 12 days following s.c. priming.

4.3.3 Per oral priming with CTA1-DD adjuvant potentiates PP Tfh and GC B cell responses

We had observed that FDC networks were more mature in neonatal PPs, which would be the site for induction of a response to an oral vaccine.[376,377] This could be a consequence of the microbial colonization of the gut in early life. Hence, we asked whether a mucosal vaccine given orally would positively affect vaccination of neonates given the more

mature FDCs in PP. To test our hypothesis, we immunized 5 day old mice per orally (p.o.) with CTA1-DD and boosted s.c. 30 days later. The induction of Tfh and GC B cells was assessed in the MLN and PPs. Whereas no induction of an immune response was recorded in MLN, PPs exhibited a significant response. We found excellent priming of neonatal systemic and local mucosal immune responses after oral immunizations with CTA1-DD adjuvant.

4.3.4 A protective oral influenza vaccine for infants

To assess the protective potential of CTA1-3M2e-DD, we primed 5-7 day old mice p.o. with the fusion protein alone or incorporated into the two types of nanoparticles (LNP and NPL). We then boosted mice twice i.n. before we analyzed the immune responses. We detected substantial increases in anti-M2e IgG-specific antibody levels in mice that received the oral priming dose. We then evaluated the mice for protection against a live challenge infection using 1xLD50 of a reassortant influenza A H3N2 virus strains X47 (A/Victoria/3/75 (H3N2) with A/Puerto Rico/8/34(H1N1)). Whereas all immunized and challenged mice demonstrated 50 to 60% survival, the most striking effect of oral priming was a faster recovery and weight gain. M2e-tetramer-specific $CD4^+$ T cells in the lung, as well as specific IgG and IgA levels in serum and BAL, were comparable between the two groups.

Our hypothesis was that a nanoparticle formulation would protect CTA1-3M2e-DD from degradation when given orally to neonates. Of note, we have previously attempted to use the CTA1-DD adjuvant orally in adult mice without any priming effect at all. We have speculated that this was because of enzymatic degradation of the adjuvant. Hence, we were concerned about whether soluble protein would have any effect in the neonates. It was, therefore, unexpected and surprising that the CTA1-3M2e-DD was effective at priming the immune response when given

orally to neonates. However, equally disappointing was that the nanoparticle formulations did not have any priming effect in the neonates and FPM2e:NPL p.o. immunizations completely failed to protect the mice against the live challenge infection.

Nevertheless, our study conveys optimisms as to the prospects of an effective mucosal vaccine for neonates and young infants. We demonstrate in the present work that the fusion protein platform provides strong maturational signals for FDCs, which contribute to the development of near normal GC reactions when tested in neonatal mice. Whereas this study did not indicate any benefit of incorporating the CTA1-3M2e-DD into nanoparticles, future attempts at selecting suitable formulations for nanoparticle delivery of the vaccine may prove otherwise. It awaits to be investigated if there are nanoparticle formulations that are effective also in neonates. It can be concluded in the present study that liposomes were more effective than porous maltodextrin nanoparticles, which hints that the formulation of the nanoparticle is critical for the immunogenicity of the fusion protein. Whereas oral priming is most effective for immune protection against enteric infections, we intend to try our vaccine concept for neonatal vaccination for protection against rotavirus infections. Noteworthy, earlier studies have evaluated oral live attenuated vaccines in a neonatal pig model with promising results, although a subcomponent vaccine based on the adjuvanted CTA1-DD:LNP fusion protein is a much bigger challenge.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The main goal of this research project was to develop a broadly protective mucosal vaccine against influenza virus infection. We did this by combining the fusion protein CTA1-3M2e-DD with two different types of nanoparticles to improve immunogenicity and protective efficacy of the fusion protein. Moreover, we explored whether immunizations of neonates could be achieved with the combined nanoparticle vaccine formulations. Whereas we have convincingly shown that co-incorporation of adjuvant active molecules and influenza specific target Ags into nanoparticles provides better local lung tissue immunity and protection in adult mice, we failed to demonstrate that the combined nanoparticles were beneficial to the development of protective immunity against influenza in neonates.

While carrying out this research project, we were challenged by several problems that had to be addressed. First of all, our fusion protein vaccine was already shown not only to stimulate strong M2e-specific serum IgG and mucosal IgA antibody responses, but also to induce high numbers of lung resident M2e-specific memory $CD4^+$ T cells.[213, 317] This meant that CTA1-3M2e-DD was already from the start a good vaccine candidate for a broadly protective influenza vaccine. Thus, we expected to improve the vaccine efficacy from an already strong standing to an even better performing vaccine by combining it with nanoparticles. To evaluate the improvement, we had to reduce the dose of Ag administered and challenge with a more aggressive influenza strain. This way, we hoped to be able to assess if there was an advantage of combining the fusion protein with the nanoparticles. Furthermore, our final goal was to

develop an oral vaccine based on the combined vector for vaccination of neonates and young children. However, we know that mucosal immune responses are compartmentalized and that oral immunization is not the most effective route for priming of a protective immune response in the respiratory tract. In this regard, we tested our neonatal vaccine under suboptimal conditions, but we believe an enteric infection would have been more appropriate. Therefore, in the next phase of the project we will test our oral CTA1-DD/LNP vaccine vector in the mouse model for rotavirus infections. Our strategy will be to design a CTA1-VP6-DD construct that will be incorporated into liposomes with a more rigid structure, which recently demonstrated promising effects in our *in vitro* experimental model. The VP6 peptide has previously been shown to stimulate strong protective immunity against rotavirus in mouse.

Another problem in the nanoparticle vaccine field is the lack of reference studies, as only few comparative studies have been done. This, unfortunately, makes generalizing conclusions problematic and the principles for how to design an optimal nanoparticle vaccine difficult. For example, at the cellular level, we lack studies that have investigated in detail how Ags carried by nanoparticles are processed and presented by DCs. It would be important to study the kinetics of these processes and whether the formulation will affect the migration of DCs to the draining lymph nodes or the priming ability of the DC in the draining lymph node. It still remains unclear whether nanoparticles that rapidly penetrate the mucosal barrier are strong inducers of mucosal immune responses or fail to stimulate local responses because of too little retention time. Alternatively, mucoadhesive nanoparticles may provide a depot of Ag for an extended loading of DCs with Ag. These considerations made it difficult to select a design of the nanoparticle that achieved features of what had previously been published as successful vaccine formulations.

Moreover, the need for simple screening systems was identified in the early phase of the project. We developed a new screening method to investigate whether a nanoparticle efficiently promoted DC-mediated priming of $CD4^+$ T cells, monitoring the increased expression of peptide bound to MHC II molecules.[319] We evaluated the performance of the nanoparticle on the basis of how effective expression of such complexes we observed *in vitro* and *in vivo*. We concluded that the density of such complexes most likely related to the ability of the DC to effectively prime the $CD4^+$ T cells in the lymph node.[319] We are convinced that this screening method can be useful in the future for screening of different type of nanoparticles.

Nevertheless, further validation of our nanoparticle formulation strategy still awaits to be done. Indeed, the validation of new biomarkers for *in vivo* identification of the most potent nanoparticle vaccines is much warranted. For example, assessments of the level of serum amyloid A (SAA) has been shown to be an early biomarker of severity of influenza infections in several mouse strains, including BALB/c, C57BL/2, Swiss-Webster and DBA.2 mice. Hence, determinations of SAA levels could be a way forward to identify effective nanoparticle vaccines. Upon treatment with oseltamivir phosphate, levels of SAA were found to be significantly decreased and high levels of SAA were associated with poor disease prognosis.[378] SSA and other biomarkers could potentially be used for early screening of different candidate nanoparticle vaccines.

In the future, we will continue developing the nanoparticle vector by replacing or adding components known to improve immunogenicity and protection against influenza virus infections. Future studies will reveal if the favorable effects of the combined fusion protein and the nanoparticle technology could be translated into a human vaccine. In particular, the physico-chemical properties of the nanoparticle appears central to improving its potential as a vaccine vector. Factors that influence the

performance of the nanoparticle are Ag load, size, charge as well as efficiency in cell targeting.[249,250,251,252,253] In this project, we tested PEGylation, which has been described as a means to prolong nanoparticle-circulation time.[280] PEG was chosen because it reduces protein aggregation owing to repulsion between PEGylated surfaces, which increases thermal stability of proteins. Moreover, PEG was shown to accelerate drainage into LNs and prolong time for Ag-uptake, improving vaccine efficacy.[282] We observed that PEGylation failed to improve the performance of the combined vector although it didn't impair the uptake of liposomes *in vitro*. By contrast, *in vivo* PEGylation decreased both the rate and extent of peptide-presentation by DCs, resulting in dramatically reduced immunogenicity. A reduction of biological potency due to PEGylation has been reported in other studies showing steric entanglement of polymer chains during the protein/receptor recognition process.[283] In future experiments, we aim to clarify the connection between the physicochemical properties of nanoparticles and their use as vaccine carriers. This work will include a careful evaluation of whether the composition of different lipids to produce the liposomes, leading to changes in liposome rigidity, will impact on DC uptake and Ag processing.

The goal of developing a universal influenza vaccine with the ability to protect against newly emerging strains requires a focus on the Ags to be included into the vaccine. This is why finding a strategy that could overcome the enormous variability and the antigenic shift of viral proteins is much needed. The solution that has been brought forward by many vaccine developers is to focus on well conserved elements, such as M2e. We may need to expand on such conserved elements and include multiple epitopes or even proteins which could confer heterosubtypic protection. Perhaps a 'cocktail' formulation would be better than a single-epitope immunogen.[379] In order to improve our vaccine candidate, we

will test the addition of the nucleoprotein (NP), which will elicit strong cytotoxic $CD8^+$ T cells.[380] In addition, instead of whole recombinant HA, we propose to include a stabilized HA stem region, as recently reported using ferritin nanoparticles, which stimulated protection against a heterosubtypic challenge infection in both mice and ferrets.[381,328]

When developing a vaccine for human use, the understanding of whether the finding in mice can be effectively translated into the human setting is of fundamental importance. This is especially true when the vaccine will be delivered intranasally. Although the overall organization of the immune system in humans and mice is quite similar, their functions may not always be the same. This topic has been reviewed by Patrick J. Haley.[382] The rodent pulmonary immune response can differ from that in humans. The composition of lymphocytes and neutrophils in mice and man is quite different: i.e. human blood is neutrophil-rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood contains more lymphocytes (75–90% lymphocytes, 10–25% neutrophils).[383] Human pulmonary alveolar macrophages (PAMs) are very effective at bacterial phagocytosis and killing, while rodents PAMs are less effective.[384] Moreover, PAMs in human show greater ability to take up particles than mouse PAMs. This could be an advantage if the vaccine is delivered into nanoparticles.[385] While mice have a significant BALT, this is largely absent in healthy humans and this could mislead us to expect an excellent performance in humans because we found the mucosal vaccine to be effective in mice.[386] Hence, a primary immunization with large numbers of antibody forming cells in the lung of mice, may not be reproduced in humans.[387,388] Moreover, the number of PPs in mice is established already at, or shortly after, birth. The number of B cell follicles and size of the PPs in humans increase with age until puberty. The presence of a microbiota is needed for the development of PP functions in mice, but whether this applies to the human gut has been incompletely

investigated.[389] The appendix, a large aggregation of lymphoid nodules at the ileocecal valve, is present at birth in humans but absent in newborn mice. It is clear that species differences exist in the structure and function of the immune system and that these differences need to be kept in mind when designing experiments with the goal of extrapolating the data for the generation of broadly protective influenza vaccines. My thesis work, though, shows that it is fruitful to further explore the combined CTA1-DD and nanoparticle concept for the improvement of mucosal vaccines and a novel influenza vaccine, in particular.

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